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<p>(54) Title: MEDICAL USE OF BROMELAIN</p> <p>(57) Abstract</p> <p>Bromelain, a mixture of enzymes derived from the stem of the pineapple, has been found to be a modulator of intracellular signal transduction, in particular a modulator of pathways in which inositol phosphates play a role, and is therefore useful in the treatment of various diseases and conditions which are mediated by these intracellular signal pathways.</p>		

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MEDICAL USE OF BROMELAIN

The present invention relates to the use of bromelain in the treatment of a variety of diseases and conditions which are mediated by intracellular signals. In particular, the invention relates to the use of bromelain in the treatment of diseases and conditions such as cancer and autoimmune diseases and as an immunosuppressive agent. Furthermore, bromelain may be used as a vaccine adjuvant.

Bromelain is the collective name for the proteolytic enzymes found in the tissues of the plant *Bromeliaceae*. Bromelain is a mixture of various moieties derived from the stem of the pineapple (*Ananas comosus*). It contains at least two proteolytic enzymes but also non-proteolytic enzymes, including an acid phosphatase and a peroxidase; it may also contain amylase and cellulase activity. In addition, various other components are also present.

Bromelain has previously been used in the treatment of a variety of conditions including inflammation and, in particular, it has been used in the treatment of diarrhoea. The use of bromelain in the treatment of infectious diarrhoea is described in WO-A-9301800, where it is suggested that bromelain works by destroying intestinal receptors for pathogens by proteolysis, and in WO-A-8801506 which teaches that bromelain detaches pathogens from intestinal receptors.

It has now been discovered that, in addition to infectious diarrhoea, bromelain is also useful in the treatment of non-infectious diarrhoea and this cannot, of

course, be explained by the mechanism of action suggested in WO-A-9301800.

5 Taussig et al, *Planta Medica*, 1985, 538-539 and Maurer et al, *Planta Medica*, 1988, 377-381 both suggest that bromelain may be of use in inhibiting tumour growth, and Taussig et al attribute this to the component or components of bromelain which have peroxidase activity but give no other explanation of mechanism. Maurer et 10 al, however, teach that bromelain is able to induce differentiation of leukaemic cell lines and that this capacity arises from the proteolytic activity. The mechanism of action of bromelain is, again, unclear. The mechanisms of action by which bromelain acts in the treatment of other conditions such as inflammation have 15 also not been satisfactorily explained.

20 In WO-A-9400147, various experiments were described which demonstrate that proteolytic enzymes and, in particular, bromelain, are capable of inhibiting secretion. The applications also disclosed that bromelain can reduce toxin binding activity and can inhibit the secretory effect of toxins such as heat labile toxin (LT) and cholera toxin (CT) and also toxins such as heat stable toxin (ST). This is in spite of the fact that ST has a 25 very different mode of action from LT and CT. These observations were explained by the fact that one component of the bromelain mixture, stem bromelain protease, appears to be capable of modulating cyclic nucleotide pathways. In addition, bromelain has also been demonstrated to inhibit secretion caused by the calcium 30 (Ca^{2+}) -dependent pathway.

LT and ST are both produced by enterotoxigenic strains of *E. coli* (ETEC). Some ETEC strains also produce pilus adhesins called colonisation factor antigens. These adhesins promote attachment of ETEC strains to the small 5 intestinal mucosa, thereby facilitating colonisation and delivery of enterotoxin. Diarrhoeal disease is ultimately dependent on production and efficient delivery of enterotoxin.

10 The enterotoxins stimulate secretion by cells by activation of signal pathways. Internal signals within cells are carried by "second messengers".

15 Every cell of the human body is constantly bombarded with various signals from its environment. Normal cells receive and process these signals which may promote growth, differentiation or death or control other functions of the cell, such as secretion of fluids in the cells of the intestinal epithelium. Therefore, signals 20 are the keys to understanding the processes in the cell which ultimately determine its fate. Signals are received through receptors with distinct biochemical activities on cell surfaces and transmit the messages further down to responder proteins. These proteins, in turn, process the signals and transduce them to other molecules within the cell. The series of biochemical 25 events that take place after the interaction of a cell with a growth factor and before the cellular response occurs is referred to as signal transduction.

30 Most cellular signals are transmitted via GTP-binding proteins, various protein kinases, protein phosphatases, enzymes that modify lipids, and second messengers such as

5 Ca²⁺ and cyclic adenosine monophosphate (cyclic AMP or cAMP). The instructions are finally interpreted in the nucleus by transcription factors that initiate gene expression and subsequent translation of cellular proteins.

10 At least three signal pathways are known to be important for secretion. One pathway employs the second messenger, cyclic AMP. Another employs the second messenger cyclic guanosine monophosphate (cyclic GMP or cGMP). These two messengers are referred to as cyclic nucleotides. The third signal pathway (Ca²⁺-dependent pathway) requires Ca²⁺ as the second messenger. WO-A-9400147 teaches that stem bromelain protease is capable of preventing diarrhoea by interfering with cyclic nucleotide and Ca²⁺-dependent pathways and thus affecting secretion.

15 The present inventor has now investigated other intracellular signal pathways and has surprisingly found that, in addition to its effect on cyclic nucleotide pathways, bromelain appears also to affect other intracellular signalling pathways, in particular pathways which are modulated by inositol phosphates, protein kinases and/or protein phosphatases.

20 Therefore in a first aspect of the present invention there is provided the use of bromelain in the preparation of an agent for modulating intracellular signalling pathways which depend on the action of inositol phosphates, protein kinases and/or protein phosphatases.

25 In the context of the present invention, inositol phosphate refers to any phosphorylated inositol molecule,

regardless of the degree of phosphorylation or the positions of the phosphate groups. Examples of inositol phosphates include phosphatidyl-4,5-biphosphate (PIP₂) and inositol-1,4,5-triphosphate (IP₃). Protein kinases and protein phosphatases refer to any molecule capable of converting an inactive form of a protein to an active form by either the addition or removal of phosphate molecules.

It has been found that bromelain is particularly useful for controlling inositol phosphate, protein kinase or protein phosphatase dependent signalling pathways which lead to the production of non synaptic extracellular signalling molecules such as vasopressin and thrombin, and particularly signalling molecules which affect growth and proliferation of cells, for example interleukins and other growth factors.

In order to grow or proliferate, normal cells require signals which are provided by growth factors produced by other cells, both nearby and in other parts of the body. This contrasts with the autonomous behaviour of the cancer cell, which is governed by its own internally generated signals. The functions of various proteins involved in cellular growth control can be intensified or modified by mutations which may change the protein structure or produce normal proteins in abnormally large amounts. Therefore, in cancer cells, the cellular apparatus for receiving and processing signals becomes defective and the cell is unable to process these signals and respond appropriately. Cells having defective growth inhibitory signals such as those arising from defective tumour suppressor genes are unable to balance

growth-stimulatory signals. Because of the defect, growth suppressing signals in the signalling cascade are not transmitted, and cells cannot curb their own proliferation. Cancer occurs when tumour suppressor genes are inactivated. Similarly, cells receiving hyper-stimulation arising from defects in the stimulatory signalling cascade, exhibit excess proliferation. Oncogenes are genes which produce a protein with altered function and their activation provides the cell with a strong, unrelenting impetus to grow. An oncogene disrupts the carefully balanced molecular controls on cell proliferation to such an extent that malignant growth ensues.

Protein tyrosine kinases such as v-src and the related v-abl protein have proved to be among the most frequently implicated proteins in experimental and human cancer. c-src is a kinase which is found in normal cells and is regulated by other kinases. This regulation is lost in v-src, found in cancer cells. The v-src kinase is persistently hyperactive as a result of a few amino acid differences between c-src and v-src proteins. Unbridled catalytic activity of the mutant protein-tyrosine kinase can have a detrimental effect on the control of cell growth. Normal cells containing c-src will only grow if stimulated to do so by growth factors; cancer cells, which contain v-src, show an acquired independence from externally supplied growth factors and, at the same time, may no longer respond to external growth-inhibitory signals. Therefore, cancer results and tumours are formed.

Protein tyrosine phosphorylation cascades (or kinase

cascades) play a significant role in regulating events throughout signal transduction. Many receptors for growth factors possess tyrosine kinase activity and, when activated, trigger the phosphorylation of multiple cellular proteins on tyrosine residues. The result of this phosphorylation process causes the target protein to gain or lose function. p21c-ras plays a critical role in mediating mitogenic and differentiating signals received from receptor tyrosine kinases (Wood et al., *Cell*, 68, 1041-1050, 1992; Thomas et al., *Cell*, 68, 1031-1040, 1992) to activation of several kinases, that include members of the protein kinase C (PKC), Raf, Mitogen-activated protein (MAP), and S6 kinase families (Cantley et al., *Cell*, 64, 281-302, 1991). These kinases can integrate signals from multiple membrane receptors.

A key element in the signalling pathway involved in transducing receptor-initiated signals to the nucleus is now recognised to be the family of mitogen-activated protein kinases (MAPk). MAPk are serine/threonine kinases that are activated by various growth factors and tumour promoters in cells. The best studied of these kinases are p42MAPK and p44MAPK (also referred to as ERK2 and ERK1 respectively, pp42mapk/erk2 and pp44mapk/erk1/mpk, also known as microtubule associated protein kinase; myelin basic protein (MBP) kinase; and RSK I and II).

Substrates of MAP kinase include pp90 and 70S rsk kinases and several transcription factors, notably Jun (Pulverer et al., *Nature*, 353, 670-674, 1991), Myc and p62TCF. Proteins that affect transcriptional activity are the most widely implicated in the cancer process.

The mechanism of activation of the MAP kinases is very complex. MAPk exists as a dephosphorylated form in quiescent cells and become activated when both tyrosine and threonine residues are phosphorylated (Boulton et al., *Cell*, 65, 663-675, 1991). In vitro, this activation is almost completely reversed if either residue is dephosphorylated (Anderson et al., *Nature*, 343, 651-653, 1990). PAC1 has recently been reported to be a MAP kinase phosphatase which inhibits MAP-kinase-regulated reporter gene expression (Ward et al., *Nature*, 367:651-653 (1994)). Phosphorylation of both tyrosyl and threonyl regulatory sites in MAP kinase is mediated by a dual specificity MAP kinase kinase (MKK or MEK). MEK is in turn regulated through phosphorylation by MAP kinase kinase kinases that include the proto-oncogene product Raf (Anderson et al., *Biochem. J.*, 277, 573-576, 1991) and MEKK, which in turn are regulated by protein kinase C (PKC).

The present inventor has now found that bromelain is capable of interfering with signalling pathways which are important for growth, in particular, signalling pathways which lead to the production of growth factors such as IL-2, platelet derived growth factor (PDGF) and insulin like growth factor (IGF).

T-lymphocytes were used as a cell model to demonstrate the mode of action of the growth-promoting mechanism of cells. The growth of T-lymphocytes is regulated through growth factor production, receptor function, cytoplasmic signal processing and gene responses in the nucleus. T-lymphocytes are a commonly used model for measurement of proliferation because of the ease of access to the cells

and the well documented role of interleukin 2 (IL-2), the T-cell growth factor which is required for growth and proliferation.

5 T-cells and, indeed, other types of cell, require stimulation in order to initiate the series of events required for proliferation. The immune system contains billions of white blood cells or lymphocytes which are divided into two classes, B lymphocytes and T 10 lymphocytes. B-cells function to protect the host from extracellular pathogens and T-cells protect the host from intracellular pathogens. B-cells and T-cells recognise distinct forms of different antigens using B-cell receptors (BCR) and T-cell receptors (TCR) respectively.

15 The activation of T cells is a complex process requiring protein tyrosine kinase activity that results in cell growth and differentiation. Activation requires recognition of antigen by the TCR and interactions with other molecules on the T cell surface with antigen presenting cells. When a T cell is presented with an appropriate antigen and the secondary co-stimulatory signal, the T-cell responds in two major ways. One is to enlarge and divide, thereby increasing the number of 20 cells that react to the antigen. The other is to secrete lymphokines or cytokines, proteins that directly inhibit the pathogen or that recruit other cells to join in the immune response. The cytokine interleukin 2 (IL-2), is a T cell growth factor which plays a pivotal role in the 25 regulation of immune responses.

30 Resting T-cells cannot normally respond to IL-2, as these cells do not express detectable high affinity IL-2

receptors on the surface of their cells. Antigenic stimulation is required for the induction of high affinity IL-2 receptor expression, and thus conferral of IL-2 responsiveness. Therefore, the initial activation signals provided by stimulation of the T cell antigen receptor (TCR), and the costimulatory signal, initiates T cell activation through induction of IL-2 production and IL-2 receptor expression. Subsequent T cell proliferation is driven by the interaction of IL-2 with its IL-2 receptor. If a T-cell receives a signal via the TCR alone, the T-cell becomes anergised or may die (called apoptosis). If a T-cell receives the co-stimulatory signal alone, the T-cell remains quiescent (or does not respond).

All the above events require tyrosine phosphorylation, as inhibitors of protein tyrosine kinases can inhibit most if not all of the later events associated with TCR stimulation (Mustelin et al., *Science*, 247, 1584-1587, 1990; June et al., *Proc. Natl. Acad. Sci. USA*, 87, 7722-7726, 1990). TCR-mediated induction of protein tyrosine kinase activity results in the tyrosine phosphorylation of many cellular proteins including the TCR zeta chain (Baniyash et al., *J. Biol. Chem.*, 263, 18225-18230, 1988), phospholipase C g1 (PLC g1) (Weiss et al., *Proc. Natl. Acad. Sci USA*, 88, 5484-5488, 1991), CD5 (Davies et al., *Proc. Natl. Acad. Sci. USA*, 89, 6368-6372, 1992), the proto-oncogene vav (Bustelo and Barbacid, *Science*, 256, 1196-1199, 1992), valosin-containing protein (VCP), ezrin (Egerton et al., *EMBO J.*, 11, 3533-3540 and *J. Immunol.*, 149, 1847-1852, 1992), ZAP-70 (Chan et al., *Cell*, 71, 649-662, 1992) and MAPk (Nel et al., *J. Immunol.*, 144, 2683-2689, 1990).

The tyrosine phosphorylation of PLC_{g1} results in its catalytic activation, resulting in the generation of the second messengers of the phosphatidylinositol (PI) pathway. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the formation of inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). These molecules in turn, function as intracellular second messengers to induce an increase in Ca²⁺ and activation of PKC, respectively. Following PKC activation, the proto-oncogene Ras is activated, Raf-1 kinase activity is increased and MAPk becomes phosphorylated. MAPk activation causes the proto-oncogenes c-fos to form a dimer with c-jun to form the transcriptional complex AP-1. The AP-1 complex binds to elements on the DNA to initiate transcription of IL-2.

Figure 1 summarizes some of the events associated with TCR activation of the PI pathway that lead to IL-2 gene transcription and IL-2 production.

Bromelain was found to inhibit the kinase cascade which is associated with growth stimulation. A factor in this signalling pathway is the ras protein of which aberrant forms are found in 25 to 30% of human tumours. Bromelain is able to block signals required for the proliferation of T-cells, probably by blocking the tyrosine phosphorylation of proteins including MAP kinase.

Because of its ability to block the tyrosine phosphorylation of MAP kinase and other proteins, bromelain is capable of acting as an anti-cancer agent since it will also block the over-production of growth factors such as platelet derived growth factor (PDGF) and

epidermal growth factor (EGF) in fibroblasts and epithelial cells.

5 In addition, because it is capable of blocking the proliferation of T-cells, it is an immunosuppressive agent which is useful in preventing the rejection by a host of a transplanted organ or in the treatment of autoimmune diseases such as diabetes mellitus, multiple sclerosis and rheumatoid arthritis. This finding is in
10 complete contrast to the teaching of WO-A-9301800 which is that compositions containing proteases such as bromelain have non-specific immunostimulant activity.

15 We have found that bromelain can, in fact, be used either to stimulate or to inhibit cytokine production depending on whether it is used to treat activated cells (such as those already receiving stimuli), or inactivated (ie quiescent or resting) cells.. It thus can be used as
20 an immunosuppression agent, e.g. in preventing tissue rejection, or as an immunostimulant, e.g. as an adjuvant to a vaccine. Bromelain can also be used to prevent or treat toxic shock by means of its ability to inhibit cytokine production and tyrosine phosphorylation. Bromelain can also be used to treat allergies.

25 As already discussed above, bromelain is a mixture of various components. Although it was taught in WO-A-9400147 that stem bromelain protease is the component of bromelain responsible for the mediation of cyclic
30 nucleotide pathways, it is not clear whether stem bromelain protease is also responsible for the action of bromelain on kinase pathways or whether some other component of the bromelain mixture could be responsible.

However, this does not affect the working of the invention since the crude bromelain mixture, at least, is capable of affecting the phosphorylation (or activation) of MAP kinase.

5

Bromelain may be administered by a variety of routes including enteral, for example oral, nasal, buccal, or anal administration or parenteral administration for example by intravenous, intramuscular or intraperitoneal injection. The oral route is, however, preferred.

10

To assist survival of bromelain through the stomach when administered orally, it may be desirable to formulate the enzyme in an enteric-protected preparation. Other orally administrable formulations include syrups, elixirs, and hard and soft gelatin capsules, which may also be enteric-coated.

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Bromelain activity is stable over a wide pH range (pH 2-9). Therefore, it may not be necessary to enteric-protect (or enteric-coat) the bromelain from the acid conditions in the stomach. It may, however, be necessary to protect the enzyme from digestion by acid proteases in the gut. Bromelain may therefore, be administered with a buffering agent, for example bicarbonate.

30

Dosage of bromelain is conventionally measured in Rorer units, FIP units, BTU (bromelain tyrosine units), CDU (casein digestion units), GDU (gelatin digestion units) or MCU (milk clotting units). One Rorer unit of protease activity is defined as that amount of enzyme which hydrolyses a standardisation casein substrate at pH 7 and 25°C so as to cause an increase in absorbence of 0.00001

per minute at 280nm. One FIP unit of bromelain activity is contained in that amount of a standard preparation, which hydrolyses a suitable preparation of casein (FIP controlled) under the standard conditions at an initial rate such that there is liberated per minute an amount of peptide, not precipitated by a specified protein precipitation reagent, which gives the same absorbence as 1 μ mol of tyrosine at 275nm. STUs, CDUs, GDUs and MCUs are as defined in the literature, as follows:

10

BTU

15

One bromelain tyrosine unit is that amount of enzyme which will liberate one micromole of tyrosine per minute under the conditions of the assay (for example, after digestion of an acid denatured haemoglobin substrate at pH 5 and 30°C).

15

CDU

20

That amount of enzyme which will liberate one microgram of tyrosine after one minute digestion at 37°C from a standard casein substrate at pH 7.0.

25

GDU

The enzyme activity which liberates one milligram (10^{-3} g) of amino nitrogen from a standard gelatin solution after 20 minutes digestion at 45°C and at pH 4.5.

$$1100 \text{ BTU/g} = 750 \text{ CDU/mg} = 1200 \text{ GDU/g.}$$

30

While the precise dosage will be under the control of the physician or clinician, it may be found that daily dosages of from 50 to 4000 GDU/day is appropriate, for

example from 100 to 1000 GDU/day. The daily dose may be given in one or more aliquots per day, for example twice, three times or four times a day. A particularly preferred dose would be 10mg/kg (giving a dose of 700mg for an
5 average adult human equivalent to 2800 BTU).

The invention will now be described by the following examples. The examples refer to the accompanying drawings, in which:

10

FIGURE 1: is a diagram illustrating the events associated with T-cell receptor activation of the phosphatidylinositol (PI) pathway which lead to IL-2 gene transcription and IL-2 production.

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FIGURE 2: is an immunoblot for the detection of MAP kinase in proteins obtained from the T-cell hybridoma GA15. GA15 were stimulated with either calcium ionophore, PMA or ionophore in combination with PMA or mock treated with PBS (unstimulated control). Cells had either been treated with bromelain or with PBS.

25

30

FIGURE 3: is an immunoblot for the detection of MAP kinase. Proteins were obtained from GA15 activated with calcium ionophore in combination with PMA or, PBS treated (unstimulated control) which had either been treated with bromelain or with PBS. Samples were tested for the presence of MAP kinase at timed intervals.

FIGURE 4: is a plot which shows the appearance over time of MAP kinase in stimulated T-cells

treated with either bromelain or PBS.

5 FIGURE 5: is an immunoblot which shows that a polyclonal antibody raised against a highly conserved peptide from MAPk (Erk 1) recognises two proteins of Mr 42000 and 44000.

10 FIGURE 6: is an immunoblot with MAPk antibody which indicates that the shifts in electrophoretic mobility normally observed on phosphorylation of protein is partially blocked in cells treated with bromelain.

15 FIGURE 7: shows the effect of bromelain treatment on IL-2, IL-4 and IFN- γ mRNA accumulation in GA15 cells in vitro. GA15 cells treated with bromelain accumulate less IL-2, IL-4 and IFN- γ mRNA when stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (500 ng/ml).

20 FIGURE 8: shows that splenic T-cells treated with bromelain produce less IL-2 when stimulated with 2C11 (α CD3e) and CD28 (α CD28).

25 FIGURE 9: shows the effect of bromelain treatment on IL-2, IL-4 and γ -INF mRNA accumulation in splenic T-cells in vitro. Splenic T-cells treated with bromelain accumulate less IL-2, IL-4 and IFN- γ mRNA when stimulated with immobilised anti-CD3e (4 μ g/ml) and soluble anti-CD28 (10 μ g/ml) mAbs.

30 FIGURE 10: shows that bromelain treatment increases the proliferation of splenic T-cells when

they are stimulated with immobilised anti-CD3e (4 µg/ml) and soluble anti-CD28 (10 µg/ml) mAbs.

5 FIGURES 11a and b : show that bromelain treatment increases the binding of both anti-CD3e (a) and anti-CD28 (b) mAbs to the cell surface of splenic T-cells as indicated by a shift in the FACS profiles to the right.

10 FIGURE 12: shows that bromelain increases the binding of anti-CD3e mAb to the surface of GA15 cells.

15 20 FIGURES 13a and b: show immunoblots of tyrosine phosphorylated proteins in splenic T-cells. Figure 13a shows that bromelain treatment induces protein tyrosine phosphorylation of 56 and 58 kDa proteins. Figure 13b shows that bromelain treatment inhibits protein tyrosine phosphorylation of a 16 kDa protein.

25 FIGURE 14: shows the effect of bromelain treatment on IL-2, IL-4 and IFN-γ mRNA accumulation in GA15 cells in vitro. Cells treated with bromelain accumulate more IL-2, IL-4 and IFN-γ mRNA when stimulated with immobilised anti-CD3e (4 µg/ml) and soluble anti-CD28 (10 µg/ml) mAbs.

30 FIGURE 15: shows the effect of bromelain on sheep red blood cell (SRBC) antibody responses in vivo. Mice that were treated with bromelain have more B cells which produce antibody directed against SRBC.

MATERIALS AND METHODS**Cell line.**

The Th0 cell hybridoma GA15 was used for experiments investigating tyrosine phosphorylation. GA15 was generated from the fusion of the thymoma BW5147 with the Th2 clone F4, specific for KLH in association with I-Ab (Fox, *Int. Immunol.*, 5, 323-330, 1993). Cells were maintained in tissue culture media (TCM) consisting of RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% fetal calf serum (Inovar Biologicals, Gaithersburg, MD), 50mM 2-mercaptoethanol, 4mM glutamine, and 50 ug/ml gentamicin.

15 Animals.

The effect of bromelain pre-treatment on IL-2 production by isolated murine splenic T cells was investigated. Male C57BL/6NCrlBR mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Young mice (3 to 4 months old) and aged mice (20 to 26 months old) were used in paired experiments. Mice found to have tumors, visible skin lesions, or significant pathology were not used. For in vivo experiments and studies investigating IL-2, IL-4 and IFN- γ mRNA accumulation, female Balb/c mice were purchased from A. Tuck and son Ltd (UK). Mice between 6 and 10 weeks of age were used.

Agonists.

Phorbol esters are structurally related to 1,2-diacylglycerol (DAG) and therefore cause activation of PKC which induces the hyperphosphorylation of Raf-1 (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 85, 8855-8859, 1988), as well as the activation of MAP

kinases (Chung et al., *Mol. Cell. Biol.*, 11, 1868-1874, 1991). Ionophore increases cytoplasmic free calcium in the cells, which in turn bind calmodulin and PKC.

5 For tyrosine phosphorylation experiments, phorbol 12, myristate 13-acetate (PMA) and calcium ionophore A23187 were used to stimulate cells. Phorbol esters and ionophore treatment of T-lymphocytes act synergistically to mimic the effect of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), and therefore reproduce many features of TCR stimulation (Truneh et al., *Nature*, 313, 318-320, 1985) such as IL-2 secretion, IL-2 receptor expression, and T cell proliferation.

15 **Isolation of splenic T-cells from mice.**
Animals were killed by cervical dislocation and spleens were removed aseptically. Single cell suspensions were prepared in tissue culture media (TCM) consisting of RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated foetal calf serum (FCS) (GIBCO Laboratories) and 2 mM L-glutamine, 5 x 10⁻⁵ M 2-ME, and antibiotics (50 mg/ml gentamicin and 10 U/ml penicillin). Erythrocytes were lysed at 5 x 10⁷ lymphocytes/ml in lysing buffer (140 mM NH₄Cl, 17 mM Tris, pH 7.2) for 2-5 min. Lysis was terminated by adding TCM and T cells were purified by incubation on nylon wool (Polysciences, Warrington, PA) for 1 h at 37°C (Julius et al., *Eur. J. Immunol.* 3: 645. (1973)). T cells were collected in the effluent and contained >90% Thy-1+ and <5% MHC class II+ cells as assessed by flow cytometry.

For measurement of IL-2 production by murine T-cells,

monoclonal antibodies (mAb) were used as agonists to mimic the molecular interactions that occur between the surface of the T-cell and the antigen presenting cell. The monoclonal antibody 2C11 (anti-CD3e chain) was used to cross-link the TCR receptor and mimic the agonistic effects of antigen peptide/major histocompatibility (MHC) stimulation. A co-stimulatory signal was provided through the CD28 molecule by using anti-CD28 mAb (37.51). Antibody ligation of the CD28 molecule and cross-linking of the TCR has been demonstrated to initiate specific signal transduction events leading to IL-2 production and T cell proliferation.

Other reagents.

Reagents were purchased from the indicated sources: sheep red blood cells (SRBC) from TCS Biologicals (Buckingham, UK); anti-CD3e-chain mAb (145-2C11) from Pharmingen (San Diego, CA); anti-CD28 mAb (PV-1) and polyclonal hamster IgG (control hamster IgG) was a generous gift from Dr. C. June (NMRI, Bethesda, MD); goat anti-hamster IgG Ab, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 all from Sigma (Dorset, UK); mouse anti-phosphotyrosine mAb (4G10) from Upstate Biotechnology (Lake Placid, NY); goat anti-mouse IgG Ab conjugated to horse radish peroxidase from Southern Biotechnology Associates (Birmingham, AL); goat anti-hamster IgG Ab conjugated to fluorescein isothiocyanate (FITC) from Cappel (Durham, NC); RNAzol B from Cinnabon/Biotecx Laboratories (Houston, TX); RNAGuard, dNTPs both from Pharmacia Biotech (St Albans, Herts); MMLV-reverse transcriptase, random primers both from GIBCO BRL (Gaithersberg, MD); Biotaq polymerase from Bioline (London, UK); crude bromelain extract (E. C. 3.4.22.4).

(batch number TAD8TK1125) from Miles Scientific (Elkhart, IN).

GA15 Cell treatment and stimulation.

5 For phosphoprotein experiments, GA15 (1×10^6 cells) were pre-treated with bromelain (15 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$, diluted in phosphate buffered saline, pH 7.4) for 30 min prior to second messenger stimulation. Cells were then washed twice by repeated centrifugation (1500 rpm, Sorvall RT 10 6000B refrigerated centrifuge; DuPont) and re-suspension in RPMI. Control cells were treated with PBS vehicle alone.

15 Cells were stimulated for various lengths of time with either calcium ionophore (1 μM), PMA (10 ng/ml) or ionophore and PMA combined. After stimulation the cells were lysed and assessed for tyrosine phosphorylated proteins as described below.

20 **Measurement of IL-2 production in splenic T-cells.**

For measurement of IL-2 production, murine splenic T cells were cultured in 96-well, flat bottom, microculture plates (Corning, Corning, NY, USA) at 10^5 cells per well. Cells were stimulated with immobilized (plate-bound) anti-mouse CD3e mAb (145-2C11) (Leo et al., Proc. Natl. Acad. Sci. USA., 84, 1374, 1987) at 100 $\mu\text{g}/\text{ml}$ and soluble anti-mouse CD28 mAb (37.51) (Gross et al., J. Immunol., 144, 3201, 1990) at 10 $\mu\text{g}/\text{ml}$. For immobilized anti-mouse CD3e mAb presentation, 145-2C11 ascites was diluted in PBS, added to microculture plates in 50 μl , incubated for 16 hours at 4°C, and then wells were washed three times in PBS. Triplicate cultures were incubated at 37°C in humidified 5% CO₂ for 24 hours prior to culture

supernatants being harvested for analysis of IL-2 activity. IL-2 activity was measured using the IL-2-dependent cell line CTL-L as previously described (Gillis et al., *J. Immunol.*, 120, 2027, 1978). Briefly, supernatants were serially diluted with 4×10^3 CTL-L cells/well in 96-well, flat bottom, microculture plates. After 24 hours, cells were pulsed with $0.5 \mu\text{Ci}/\text{well}$ [^3H]thymidine for an additional 16 hours. Cells were harvested and proliferation assessed by liquid scintillation counting. Units were calculated using recombinant murine IL-2 (Pharmingen, San Diego, CA, USA) as the standard.

In experiments measuring IL-2, IL-4 and IFN- γ mRNA accumulation and in FACS analysis cells were incubated at 37°C for 30 min in RPMI 1640 containing 50 $\mu\text{g}/\text{ml}$ bromelain at 10^7 cells/ml. Mock treated cells were incubated with an equal volume of phosphate-buffered saline (0.1 M, pH 7.4; PBS) (diluent for bromelain). Following incubation, cells were washed 3 times in RPMI 1640 and then resuspended in TCM.

Cell culture for mRNA determination.

In experiments investigating the accumulation of cytokine mRNA, GA15 hybridomas were cultured in 24 well, flat-bottom plates (Nunc, Roskilde, Denmark) at 2.5×10^6 cells per well in a 2 ml culture volume for 4 h. Splenic T cells were cultured in 6 well, flat-bottom plates (Flow Laboratories, McLean, VA) at 5×10^6 cells per well in a 1 ml culture volume for 24 h. In experiments investigating proliferation, splenic T cells were cultured in triplicate in 96 well, flat-bottom plates (Nunc) at 10^5 cells per well in a 200 μl culture volume

for 36 h. These cultures were pulsed with 0.5 μ Ci of [3 H]TdR 16 h prior to harvesting onto glass fibre filters and counting incorporated [3 H]TdR. All reagents were diluted in TCM, except for immobilised anti-CD3e mAb which was prepared by diluting the mAb in PBS, adding to culture plates to cover the bottom of wells, incubating for 16 h at 4°C and then washing the wells 3 times with PBS. All cells were incubated at 37°C in humidified 5% CO₂.

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Flow cytometry.

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Prior to staining, cells (10^6) were incubated in 1 ml fluorescent activated cell sorting (FACS) buffer (1% heat-inactivated FCS in PBS, 0.02% NaN₃) containing 50% filtered horse serum for 30 min on ice and then washed once with FACS buffer. Cells were stained in 100 μ l FACS buffer for 30 min on ice with either anti-CD3e mAb, anti-CD28 mAb or hamster IgG control Ab (all at 10 μ g/ml) and washed once with FACS buffer. Ab specifically bound to cell surfaces was detected by incubating the cells in 100 μ l FACS buffer for 30 min on ice with FITC-conjugated anti-hamster IgG (10 μ g/ml) and then washing once in FACS buffer. Cells were resuspended in 1% paraformaldehyde (diluted in FACS buffer) and stored at 4°C. Cells were analysed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Gates were set on lymphocytes using forward and side scatter and data were plotted on a log scale.

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PCR analysis of cytokine mRNA.

IL-2, IL-4 and IFN- γ mRNA accumulation was measured using an adaption of a previously described semi-quantitative RT-PCR assay (Svetic et al, J. Immunol. 147: 2391-2397

(1991)). Briefly, RNA was isolated from cells and splenic tissue (Chomczynski and Sacchi, Anal. Biochem. 162: 156-159 (1987)) using RNAzol B according to the manufacturers instructions (Cinna/Biotecx Laboratories), and the mRNA was reverse transcribed by standard procedures (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (1989)) using 3 µg of total RNA recovered from samples in a final reaction volume of 25 µl. PCRs were performed in duplicate for each sample in a final volume of 25 µl using 2.5 µl of reverse transcribed mRNA sample as cDNA template. Oligonucleotides specific for IL-2, IL-4, IFN-γ and the housekeeping gene hypoxantine-guanineohosphoribosyl transferase (HPRT) and other components of the PCRs were as previously described (Svetic et al (1991) *supra*).

All PCRs consisted of a denaturation step at 95°C for 1 minute, an annealing step at 55°C for 1 minute and an extension step at 72°C for 2 minutes. The number of PCR cycles used to ensure that amplification was terminated when amplified product could be detected, but was well below saturating concentrations, was determined for each cytokine and RNA sample analysed. PCR products were detected by size-fractionating amplified DNA by agarose-gel electrophoresis and transferring the DNA to Hybond N+ nylon membrane according to the manufacturers instructions (Amersham, Buckinghamshire, UK).

Amplified cytokine mRNA was revealed using cytokine-specific oligonucleotides (Svetic et al (1991) *supra*) labelled with horse-radish peroxidase (HRP) and reacted with the ECL chemiluminescence detection system, as

described by the manufacturer (Amersham). Specific signals were recorded on autoradiographic film (Kodak, Rochester, NY) and analysed on a Sharp JX-3F6 computing densitometer (Sharp, Japan) using Phoretix 1-D software (Phoretix International, Newcastle, UK).

The intensity of signals generated by HPRT products was used to ensure even loading of target cDNA into PCRs. Generally, signals generated by HPRT products were within 10% of the intensity of corresponding signals from samples isolated from cells stimulated in the same way from the different treatment groups. The intensity of signals generated by cytokine products was calculated relative to signals generated by HPRT products for each sample.

Immunoblotting of cells.

Cell phosphotyrosine blots were performed as described by Thomas et al. (Cell, 68, 1031-1040, 1992). Briefly cell lysates from 1×10^6 GA15 cells were prepared by lysing the cells in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 4 mM EDTA, 1% Triton X-100, 4 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, 10 μ g/ml leupeptin) for 30 min with continual rotation. Lysates were clarified (14,000 xg for 2 min), and suspended in SDS-PAGE sample buffer and boiled for 5 min. Samples containing equal amounts of protein were resolved on a 12.5% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred to nitrocellulose (BioRad), and unoccupied binding sites were blocked in blocking solution containing 5% bovine serum albumin (Sigma, fraction V; BSA), 0.05% NP-40, 170 mM NaCl and 50 mM Tris (pH 7.5). Immunoblots were incubated with

anti-phosphotyrosine monoclonal antibody (4G10; IgG2bk) at 1 μ g/ml (Upstate Biochemical Industries, NY) to detect tyrosine phosphorylated proteins. Bound monoclonal antibody was detected by horseradish peroxidase-conjugated goat anti-mouse IgG. Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham Corp., Arlington Heights, IL) and specific signals were recorded on autoradiographic film.

10 Immunoblotting of separated proteins for detection of MAP kinase was conducted using rabbit anti-rat MAP kinase R2 (erk1-CT) polyclonal IgG, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. The anti-MAP kinase antibody used recognises the 42 kda, 43 kda and 44 kda MAP kinases encoded by the erk1 gene, mapk gene and mpk gene, respectively.

15 20 Analysis of protein tyrosine phosphorylation following stimulation with α CD3e and α CD28 mAb.

Cells were suspended at 5-10 x 10⁷ cells/ml in RPMI 1640 at 37°C 5 min prior to stimulation. PMA and calcium ionophore A23187 were used at 20 and 500 ng/ml, respectively. Cross-linking of CD3e and CD28 mAbs was carried out by incubating T cells on ice for 30 min in the presence of 10 μ g/ml of each mAb. After excess antibodies were washed away, cells were suspended at 5-10 x 10⁷ cells/ml in RPMI 1640 (all at 4°C). Cross-linking was performed at 37°C with 10 μ g/ml goat anti-hamster IgG. Cells were stimulated for times indicated in figure legends and the text. Stimulation was terminated by the addition of ice-cold stop buffer, yielding a final concentration of 0.5% Triton X-100, 25 mM Tris, pH 7.2,

75 mM NaCl, 400 mM EDTA, 10 mM sodium fluoride, 400 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 74 mg/ml leupeptin, 740 mM PMSF and 74 µg/ml aprotinin. After lysis at 4°C, samples were centrifuged at 12,000 rpm (13,200 g), 4°C for 15 min. Postnuclear supernatants were collected and an equal volume of 2 x SDS-PAGE sample buffer (50mM Tris, pH 7, 700 mM 2-ME, 50 % (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) was added. Proteins were solubilised at 100 °C for 5 min and resolved by SDS-PAGE.

Sheep red blood cell assay.

Mice received 3 x 200 µl intravenous (i.v.) injections of 200 µg bromelain or and equal volume of 0.9% NaCl (diluent for bromelain) on days 1, 4 and 6 of the experiment. Both treatment groups received either a 100 µl intraperitoneal (i.p.) injection of 10⁷ SRBC or an equal volume of 0.9% NaCl (diluent for SRBC) on day 4 of the experiment. Mice were killed on day 7, their spleens were removed and splenocytes isolated as described in the cell preparation section. The number of B cells secreting antibodies specific for the SRBC antigen was determined by an assay based on the original method of Jerne and Nordin, described by Weir (1986). Briefly, assays were performed in 160 µl, consisting of 5 x 10⁵ splenocytes, 6 x 10⁶ SRBC and 1:27 guinea pig complement in RPMI 1640. The reaction mix was placed in a chamber created by joining two glass slides together with double sided tape and then sealed with wax. Samples were incubated at 37°C for 1 h, prior to counting plaque forming cells (PFC) (i.e., B cells secreting Ab specific for SRBC).

RESULTSEXAMPLE 1

5 Effects of bromelain on ionophore and phorbol ester-induced tyrosine phosphorylation in GA15.

The effects of bromelain pre-treatment of T-cells on second messenger stimulation of cellular substrates of tyrosine kinases were assessed. Immunoblots using 10 specific anti-phosphotyrosine antibodies revealed tyrosine phosphorylation of several protein bands after 5 min stimulation with PMA, ionophore, or PMA and ionophore combined (PMA + Ca). A major protein band of approximately 42 kda was the most visible. A synergistic effect of PMA + Ca, increased the intensity 15 of the phosphorylated band (Figure 2).

20 The effect of bromelain on tyrosine phosphorylation was assessed by its ability to inhibit the presence of the 42 kda band, as detected by anti-phosphotyrosine immunoblots. Bromelain pre-treatment of the GA15 cells blocked the tyrosine phosphorylation of the 42 kda protein when stimulated by all second messenger agonists. This band was not detected by phosphotyrosine 25 immunoblotting. Because of the synergistic effect of PMA + Ca combined, all experiments were subsequently conducted with this combination.

30 We next examined the kinetics of phosphorylation and determined whether the ability of bromelain to inhibit tyrosine phosphorylation at five minutes was a function of time or whether bromelain completely inhibited tyrosine phosphorylation. We saw that bromelain did not

completely inhibit phosphorylation, but delayed tyrosine phosphorylation (Figure 3). The kinetics of phosphorylation were then quantitated by densitometric measurements of autoradiograms (see Figure 4). Despite 5 the delay in tyrosine phosphorylation, phosphorylation of this band did not reach the same intensity as that observed in untreated controls and was completely dephosphorylated by 180 minutes (data not shown).

10 Inhibition of some other as yet unidentified proteins, in addition to the 42 kda protein, was also observed when cells were stimulated with second messenger agonists. Bromelain however, did not affect the overall phosphorylation of abundant cellular phosphoproteins in 15 unstimulated cells, as examined by anti-phosphotyrosine immunoblotting.

Identification of the major 42 kda phosphorylated band, As mentioned above, several members of the MAPk family 20 are phosphorylated on tyrosine residues following treatment with growth factors, and also by phorbol esters and calcium ionophores. Since the molecular weights of MAPk described in the literature correspond to the major protein band tyrosine phosphorylated in our experiments, 25 we hypothesised that the 42kda protein observed in GA15 cells was MAPk. We therefore used anti-MAPk antibodies to detect MAPk in GA15 cells.

Figure 5 shows that a polyclonal antibody raised against 30 a highly conserved peptide from MAPk (Erk 1) recognises two proteins of Mr 42,000 and 44,000 (presumably erk 1 and erk 2, respectively). The 42 kda protein displays an electrophoretic mobility similar to the 42 kda

phosphotyrosine-containing protein detected in stimulated T- cells. The 44 kda band also correlates with a protein which is tyrosine phosphorylated independent of stimulation with PMA plus ionophore. A protein of 48 kda and other low molecular weight proteins were also detected, however as these bands were also detected in control immunoblots with no anti-MAPk addition, reactivity was thought to be non-specific.

10 Immunoblotting with anti-MAPk antibodies revealed similar intensities in all samples tested, indicating that the reduction in intensity of tyrosine phosphorylation observed in bromelain-treated cells was not due to differences in the level of protein present.

15 **Electrophoretic mobility.**
We also investigated whether the 42 kda band phosphorylated in our experiments was MAPk by detecting phosphorylated MAP kinase through its characteristic retardation in electrophoretic mobility. Cell lysates 20 were examined by immunoblot analysis with anti-MAPK antibody.

25 Immunoblotting with MAPk antibody indicated a shift in electrophoretic mobility consistent with that normally observed upon phosphorylation of MAPK. This shift in mobility was also partially blocked in cells treated with bromelain (Figure 6).

30 **Effect of bromelain on IL-2, IL-4 and IFN- γ mRNA production in GA15 in vitro.**

Above it was shown that treatment of the GA15 T cell hybridoma with bromelain results in alterations in

tyrosine phosphorylation when cells were stimulated with PMA and calcium ionophore A23187. In particular, bromelain markedly reduced tyrosine phosphorylation of a 42 kDa protein called MAP kinase. To determine if alterations in tyrosine phosphorylation patterns induced by bromelain resulted in any functional changes, we measured cytokine mRNA accumulation in GA15.

The effect of bromelain on IL-2, IL-4 and IFN- γ production was tested by measuring the accumulation of mRNA encoding for these cytokines after 4 h of culture using a RT-PCR assay. GA15 cells were stimulated with PMA and A23187. This combination of stimuli (PMA and A23187) directly activates cell signalling pathways and therefore bypasses all cell surface molecules. The relatively short culture period (4 h) used was to ensure that cytokine mRNA accumulated was a result of the specific stimuli used, rather than an autocrine effect of newly synthesised cytokine.

Cytokine mRNA was not detected in cells cultured in TCM alone. The accumulation of all cytokine mRNA species following stimulation with PMA and A23187 was consistently lower in GA15 cells treated with bromelain, compared with control (PBS) cells (Figure 7). The difference in cytokine mRNA accumulation was most apparent for IL-4, which was significantly lower ($n=3$, $p<0.05$) in bromelain treated cells. Because tyrosine phosphorylation of MAP kinase is associated with cytokine gene transcription (Pelech, Curr. Biol. 3: 513 (1993)), we hypothesise that the reduction in cytokine mRNA accumulation resulted from reduced tyrosine phosphorylation of MAP kinase.

Effect of bromelain on IL-2 production in splenic T-cells.

We next investigated whether bromelain could affect cytokine production in normal T-cells isolated from mouse (murine) spleens when stimulated via cell surface molecules. The first combination of stimuli (PMA+A23187) directly activates cell signalling pathways and therefore bypasses all cell surface molecules. A combination of immobilised α CD3e and soluble anti-CD28 mAbs would provide a primary signal through the T-cell receptor (via the CD3e-chain) and a co-stimulatory signal through CD28 to activate cell signalling cascades. Both combinations of stimuli (PMA+A23187; α CD3e+ α CD28) have previously been shown to provide optimal T-cell activation (Truneh et al., *Nature*, 313:318-320 (1985); Linsley and Ledbetter, *Ann. Rev. Immunol.*, 11:191 (1993)) however by a different mechanism ie, direct and indirect action respectively.

The secretion of IL-2 into culture supernatants by naive murine splenic T-cells was measured. T cells were stimulated for 24 hours with immobilised anti-CD3e mAb in the presence of anti-CD28 mAb, as described above. T-cells were cultured in the absence of antibody or in the presence of anti-CD28 or anti-CD3e alone to serve as controls.

Figure 8 shows results from a single experiment. T cells cultured in the absence of antibody or with anti-CD28 alone did not produce detectable levels of IL-2 (data not shown). Similarly, immobilised anti-CD3e alone, produced barely detectable levels of IL-2. As both a primary signal through the TCR and a co-stimulatory signal are required for optimal T-cell activation, T cells were

cultured in the presence of both antibodies.

The combination of anti-CD28 and anti-CD3e in cultures resulted in a substantial increase in IL-2 production by 5 T-cells. A difference in IL-2 production, following stimulation, was observed between young and aged mice. This phenomena has been widely reported in the literature (reviewed by Thoman and Weigle, 1989) . Again, as observed earlier in GA15 experiments, T cells that were 10 pre-treated with bromelain, produced markedly less IL-2 than T-cells that were pre-treated with PBS alone. A similar reduction was noted in both aged and young mice. This effect was observed when T cells were optimally stimulated with a primary signal through the TCR and a 15 costimulatory signal via the CD28 accessory molecule. The effect of bromelain did not appear to be aged related, because T cells from both young and aged mice exhibited reductions in IL-2 production.

20 **Effect of bromelain on IL-2, IL-4 and IFN- γ mRNA production in splenic T-cells in vitro.**

We next investigated whether bromelain-induced alterations in tyrosine phosphorylation patterns could affect other cytokines produced by splenic T-cells. We 25 investigated the effect of bromelain on IL-2, IL-4 and IF- γ production by measuring the accumulation of mRNA encoding for these cytokines. T cells were cultured for 24 h in the presence of either media alone or immobilised anti-CD3e and soluble anti-CD28 mAbs combined. Cytokine 30 mRNA was not detected in cells cultured in TCM alone (data not shown).

The accumulation of all cytokine mRNA species following

stimulation was consistently lower in bromelain-treated T cells, compared with controls (Figure 9). The differences in cytokine mRNA accumulation was most apparent for IL-4, which was significantly less ($n=3$, $p<0.05$) in bromelain-treated cells stimulated with anti-CD3e and anti-CD28 mAbs.

Effect of bromelain on splenic T-cell proliferation in vitro.

Given the reduced cytokine mRNA accumulation observed in bromelain pretreated splenic T cells and the importance of these cytokines, particularly IL-2 and IL-4, for T cell proliferation, bromelain would be expected to cause a reduction in T cell proliferation. Therefore, we investigated the effect of bromelain on T cell proliferation by measuring ^3H -TdR incorporated in cells stimulated with anti-CD3e and anti-CD28 mAbs. Surprisingly, bromelain pretreatment of T cells caused a significant increase ($n=6$; $p<0.05$) in T cell proliferation, instead of decreased proliferation, as expected (Figure 10). One possible explanation for these contradictory effects is that bromelain might cause reduced cytokine production, but increase responsiveness to growth factors, such as IL-2 and IL-4 (possibly by modifying cell surface receptors for such growth factor or by increasing expression via effects on signalling cascades), in T cells.

Effect of bromelain on splenic T cell surface molecules.

Because bromelain has previously been demonstrated to remove specific T-cell surface molecules (Hale and Haynes, *J. Immunol.*, 149:3809-3816 (1992)) we investigated if bromelain was removing CD3e and CD28

5 molecules on bromelain-treated splenic T cells by flow cytometry. The mAbs used in these studies were the same used in stimulation assays described above. We observed that the binding to CD3e on the surface on splenic T
10 cells was consistently higher when cells were treated with bromelain (Figure 11a). An increase in binding of anti-CD28 mAb to CD28 (expressed at detectable levels on splenic T cells) was also found (Figure 11b). These results indicate that the removal of cell surface molecules by bromelain treatment was not responsible for the reduced cytokine mRNA accumulation observed, although the increased binding of mAbs to CD3e and CD28 molecules may have contributed to the increased proliferation of bromelain-treated cells.

15 Effect of bromelain on GA15 cell surface molecules.
20 Since we observed an increase in binding of α CD3e to splenic T-cells following bromelain treatment, we also investigated whether bromelain affected CD3e and CD28 molecules on GA15 cells. The mAbs used in these studies were the same used in stimulation assays described above. Again, the binding of mAb to CD3e on the surface of GA15 appeared to increase slightly (indicated by the increase in mean fluorescence intensity (profile shifting to the right)), following bromelain treatment (Figure 12). This result is consistent with that observed earlier with splenic T-cells. The increase observed was a specific event because a control mAb showed no increase in binding above background levels in both bromelain and PBS treated
25 GA15. It is unlikely that the increase in binding of the anti-CD3e mAb to the surface of GA15 resulted from increased expression of CD3e because cells were only treated with bromelain for 30 min at 37°C and all
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subsequent staining procedures were conducted at 4°C, where very little cellular activity would be expected. It is possible that increased binding of the mAb to CD3e could have resulted from bromelain modifying the molecule to expose more antigenic determinants.

Effect of bromelain on tyrosine phosphorylation in normal murine splenic T cells in vitro.
Earlier we investigated the effect of bromelain on tyrosine phosphorylation in GA15 cells. We next investigated the effect of bromelain treatment on tyrosine phosphorylation in normal murine T cells. T cells were isolated from the spleens of healthy Balb/c mice and stimulated with either PMA and A23187 or immobilised anti-CD3e and soluble anti-CD28 mAbs as described for GA15 cells above. Splenic T cells were isolated and then cultured for 48 h in TCM containing 5 ng/ml PMA as described by Vandenberghe et al, J. Exp. Med. 175: 951-960 (1992)), prior to stimulation for analysis of tyrosine phosphorylation. The reason for this pre-culture in PMA is that increased tyrosine phosphorylation is very difficult to detect in resting T cells (Vandenberghe et al, (1992) *supra*).

A different pattern of tyrosine phosphorylation was observed in splenic T cells than that previously observed in GA15 (Figure 13a and 13b). Bromelain inhibited the tyrosine phosphorylation of a low molecular weight (approximately 16 kDa) protein, regardless of whether cells were stimulated or not (Figure 13b). The 42 kDa tyrosine phosphorylated MAP kinase band previously observed in GA15 was not detected. A more sensitive assay for measuring MAP kinase activity may be required

for these cells. Another striking difference between tyrosine phosphorylation patterns in splenic T cells and GA15 was that bromelain treatment resulted in tyrosine phosphorylation of proteins (Figure 13a), whereas in GA15 cells it had had an inhibitory effect, following stimulation. When T cells were stimulated with either PMA and A23187 or anti-CD3e and anti-CD28 mAbs, a prominent 56 kDa tyrosine phosphorylated protein was observed. In addition, a slightly larger 58 KDa tyrosine phosphorylated protein was also detected in these cells. Tyrosine phosphorylation of these proteins was not detected unless T cells were treated with bromelain. These proteins were tyrosine phosphorylated between 2 and 5 min after stimulation with anti-CD3e and anti-CD28 mAbs, and remained phosphorylated for at least 30 min after stimulation (Figures 13a and 13b). Because phosphorylation of some proteins is associated with activation of cellular responses, the ability of bromelain to induce tyrosine phosphorylation in T cells suggests that bromelain may activate these cells. Earlier results obtained which investigated the effect of bromelain on splenic T-cell proliferation, would suggest that these cells were activated by bromelain.

25 EXAMPLE 2

In the previous example we noted that bromelain caused decreased phosphorylation of proteins, particularly MAP kinase, in GA15 cells (a T-cell hybridoma). We also observed that bromelain treatment of GA15 cells resulted in decreased IL-2, IL-4 and IFN- γ when stimulated with phorbol ester and ionophore. In addition we also observed that bromelain treatment of normal murine splenic T-cells

caused a similar reduction in IL-2, IL-4 and IFN- γ when provided with a more physiological stimuli ie, α CD3e and α CD28 which provides a signal via the T-cell receptor and a co-stimulatory signal via CD28 respectively. Taken 5 together, this data suggest that decreased tyrosine phosphorylation correlates with a reduction in cytokine production. However, data obtained in Figure 13a which shows increased tyrosine phosphorylation of proteins, and the observed increase in proliferation of the splenic T-cells (Figure 10) would suggest that bromelain is also 10 stimulating or activating the T-cells.

We conducted additional experiments and investigated the effect of bromelain on cytokine mRNA produced following 15 stimulation of GA15 cells with α CD3e and α CD28 (earlier experiments in example 1 investigated the effect of bromelain and PMA+A23187 on cytokine production).

Interestingly we did observe an increase in cytokine mRNA 20 production when cells were treated with bromelain and stimulated with anti-CD3e and anti-CD28 mAbs (Figure 14).

This pattern of cytokine mRNA accumulation is the opposite to that observed in example 1, whereby bromelain 25 reduced cytokine mRNA accumulation in GA15 cells when stimulated with phorbol ester plus ionophore.

Again, the difference in cytokine mRNA accumulation was most apparent for IL-4, which was significantly higher 30 (n=3, p<0.05) in bromelain treated cells stimulated with anti-CD3e and anti-CD28 mAbs (earlier, bromelain induced significantly lower (n=3, p<0.05) IL-4 mRNA accumulation in GA15 cells stimulated with PMA+A23187). One possible

explanation for the different results described above is that in GA15 cells, bromelain treatment inhibits signalling pathways directly stimulated by PMA+A23187, but can augment alternate signalling cascades associated 5 with cell surface molecules, such as CD3e and CD28.

The effect of bromelain in mice *in vivo*.

The data presented thus far suggests that bromelain could have multiple effects on T cells (both stimulatory and 10 inhibitory, depending on the cell type studied). We have shown different effects on tyrosine phosphorylation patterns between normal splenic T cells (mainly naïve or inactivated T cells) and the T cell hybridoma GA15 (representative of an activated T cell). In addition, 15 when ligands for cell surface molecules were used to stimulate cells (i.e., anti-CD3e and anti-CD28 mAbs), bromelain treatment increased cytokine mRNA accumulation in GA15, but reduced cytokine mRNA in normal splenic T cells, despite causing an increase in proliferation in 20 these cells.

Given the differences observed between cells cultured *in vitro*, we next investigated the effect of bromelain on T cell function in intact animals. The effects of 25 bromelain on T cell activation in a well described *in vivo* model of T cell-dependent antibody responses to sheep red blood cells (SRBC) (Weir, Handbook of Experimental Immunology, 1-4, 4th edn. Blackwell Scientific Publications, Oxford, UK (1986)) was 30 investigated. This assay directly measures the number of B cells which produce Ab specific for SRBC (i.e., plaque forming cells (PFC)), following immunization with this antigen. This response is dependent on SRBC-specific T

cells proliferating and the production of cytokines, particularly IL-4.

Mice immunised with saline (control) produced very few PFC, regardless of whether they were pre-treated with bromelain or saline (control) (Figure 15). This indicated that bromelain treatment was not causing the spontaneous production of PFC. In mice immunised with SRBC, the administration of bromelain caused a significant increase ($n=11$, $p<0.05$) in PFC, compared with control animals (Figure 15). One possible explanation for this result is that bromelain treatment caused increased proliferation of SRBC-specific T cells (similar to the increased proliferation seen in experiments conducted with splenic T-cells in vitro, Figure 10), resulting in enhanced T cell help for SRBC-specific Ab production by B cells. However, we cannot rule out the possibility that bromelain was directly stimulating B cells or some other cell involved in the antibody response. Regardless of the precise mechanism involved, this result suggests a novel application for bromelain as an adjuvant.

DISCUSSION

Bromelain has been reported to inhibit the secretory effects of various second messengers such as cyclic AMP, cyclic GMP and Ca^{2+} in intestinal cells. The mechanism of action of bromelain is unknown, but is thought to act proximal to the accumulation of cyclic nucleotides in the cells.

In view of the effects of bromelain on intestinal cells,

and the role that second messengers and protein phosphorylation play in these events, we tested whether bromelain could inhibit signal transduction systems in other cells. Specifically, we examined the effect of bromelain on the inositol pathway and tyrosine phosphorylation required for the production of cytokines (IL-2, IL-4 and IFN- γ) and proliferation in T-cells. We hypothesised that if bromelain was affecting signal transduction in cells, then this inhibition would be accompanied by a failure of T-cells to produce cytokines, major autocrine growth factors and proliferation.

The results indicate that bromelain can either inhibit or stimulate tyrosine phosphorylation of proteins when stimulated by phorbol esters and calcium ionophores, and antibodies directed against surface molecules. One of the affected proteins we identified, is presumed to be MAPk (mitogen activated protein kinase). Several observations support our presumptive identification:

1) MAPk becomes phosphorylated upon stimulation of T-cells with phorbol esters and calcium ionophores, and is also phosphorylated upon ligation of the T-cell receptor and a costimulatory molecule.

2) A 42 kda protein, which corresponds to the literature value of 42 kda is phosphorylated in GA15 cells upon stimulation with phorbol ester and ionophore. This protein was not phosphorylated or phosphorylation was markedly reduced when cells were treated with bromelain.

3) Immunoblotting with specific anti-MAPk

antibodies resulted in recognition of a 42 kda protein band.

5 4) Immunoblotting revealed that bromelain caused retardation of electrophoretic mobility of the 42 kda protein, which is characteristic of phosphorylation of MAPk.

10 Because of the inhibitory effect of bromelain on tyrosine phosphorylation and MAPk, we postulated that this inhibition would be associated with impaired signal transduction. MAPk is important for IL-2 production, as it is able to phosphorylate other proteins such as c-Jun, which is required for the initiation of IL-2
15 transcription. Therefore we tested the ability of bromelain to inhibit IL-2 production in murine splenic T cells. Interestingly, T cells that were pre-treated with bromelain, produced markedly less IL-2 than T-cells that were pre-treated with PBS alone, following stimulation
20 with anti-CD3e mAb and anti CD28 mAb.

25 The precise mechanism of action of bromelain is unknown. It is possible that it inhibits tyrosine phosphorylation by inhibiting tyrosine kinase activity, or by stimulating a phosphatase that results in dephosphorylation of proteins (for example activating CD45).

30 Irrespective of the mechanism of action, modulators of protein tyrosine kinase activity have been proposed to have immunosuppressive properties (June et al., Proc. Natl. Acad. Sci. USA, 87, 7722-7726, 1990). Herbimycin A, has been shown to inhibit the early biochemical events of antigen receptor-stimulated T-cell activation and is

correlated with inhibition of protein tyrosine kinase activity. Herbimycin, however cannot inhibit effects of phorbol esters and ionophore stimulation. The effects of bromelain are different to those reported previously for herbimycin A, in that bromelain can inhibit effects of T-cell receptor stimulation (demonstrated in antibody ligation experiments) and effects of second messengers (phorbol ester and ionophore).

Bromelain has different effects from rapamycin which is also reported to have immunosuppressive properties. Unlike rapamycin, bromelain inhibits tyrosine phosphorylation of MAP kinase (Chung et al., Cell 69, 1227-1236, 1992). Cyclosporin A, another immunosuppressant inhibits calcineurin, again distinct from the action of bromelain.

Because of the ability of herbimycin and rapamycin to inhibit protein tyrosine activity and signal transduction, they are proposed to be of use as an immunosuppressive agents. In this study bromelain has been shown to inhibit tyrosine phosphorylation in T cells, it can therefore be predicted that bromelain too may have potential as an immunosuppressive agent.

Herbimycin A has also been shown to induce differentiation in a number of cell lines and in one case this has been correlated with inhibition of protein tyrosine kinase activity (Kondo et al., J. Cell. Biol., 190, 285-293, 1989).

The results discussed above indicate a number of potential applications for bromelain. The fact that

bromelain effects tyrosine phosphorylation patterns in T cells indicates that cellular events resulting from this signalling mechanism could be manipulated by the use of bromelain. In addition, given the various effects of bromelain on different mammalian cell types and the importance of tyrosine phosphorylation in all of these cells, bromelain could modulate cellular events resulting from signalling mechanisms in a wide range of cells.

In addition to the effect of bromelains on tyrosine phosphorylation, we found that bromelain treatment of T cells could modify cell surface receptors to increase binding to ligands. Previously, bromelain was only thought to affect T cells by cleaving surface receptors (Hale and Haynes, (1992) *supra*). Therefore, we believe that we have found two more mechanisms (in addition to cleaving cell surface molecules) by which bromelain effects T cells (i.e., modifying tyrosine phosphorylation and modifying specific cell surface receptors to increase binding to their physiological ligands).

Based on these results it is believed that bromelain could be used to modify the following cellular processes;

Modification of cytokine production.

We have direct evidence that bromelain can stimulate (Figure 14) or inhibit (Figures 7, 8 and 9) cytokine production in T cells. Potential applications for using bromelain to stimulate cytokine production include as an immunoenhancer in immunocompromised individuals or those infected with a parasite/pathogen, and as an adjuvant for vaccines (direct evidence for this in Figure 15) or chemotherapies. Potential applications for using

bromelain to inhibit cytokine production include as an immunosuppressor to prevent tissue rejection following transplantation and to prevent autoimmune responses. Also bromelain may have an application for preventing toxic shock (inflammatory cytokine production by an individual is an important contributor to toxic shock). The phosphorylation of proteins, including MAP kinase is thought to be important in toxic shock. Tyrosine kinase inhibitors have been demonstrated to inhibit septic shock in vivo (Novogrodsky et al, Science. 264: 1319-1322 (1994)). Similarly, bromelain may be used to prevent allergic reactions. Inflammatory cytokines and other cellular products such as histamine are released from cells following exposure to allergens. The signalling cascades which lead to secretion of inflammatory products from cells involve tyrosine phosphorylation.

Stimulation of T cell proliferation or differentiation. We have direct evidence that bromelain can stimulate normal splenic T cell proliferation (Figure 10). Given the fact that as a naïve T cell proliferates it can differentiate into a specific type of cytokine producing cell, we believe that bromelain may also be able to effect this differentiation process. The potential application for using bromelain to stimulate T cell proliferation are the same as for increasing cytokine production. However, in addition to increased T cell proliferation which would lead to more cells capable of producing cytokines, there will also be more cells to provide cell-cell interactions, which, in addition to cytokine production, are also a vital component of an immune response. Another advantage of promoting cellular differentiation is in leukemias, or T-cell cancers,

whereby disease results because of an increased population of undifferentiated T-cells (N.B. we do not anticipate that bromelain would stimulate proliferation of abnormal T cells, as data generated in GA15 cells, a T-cell hybridoma, demonstrate that bromelain inhibits proliferation of these cells).
5

Prevention of T cell death.

One possible explanation for bromelain causing an increase in normal T cell proliferation (Figure 10) could be that it prevents programmed cell death (apoptosis). A decrease in cell death would lead to more cells becoming available to incorporate ^3H -TdR (used to measure proliferation). Apoptosis is specific event whereby 10 cells are stimulated to destroy their own DNA and die. It is an essential event in most immune responses (to prevent the accumulation of too many cells), but can also have immunosuppressive consequences in some instances, such as in HIV infection and ageing (i.e., too many cells 15 die and not enough are left to combat infection) (Perandones et al, J. Immunol. 151: 3521-3529 (1993)). Because the initiation of apoptosis is dependent on 20 specific cell signalling events, our evidence that bromelain effects tyrosine phosphorylation (Figure 13a), which may be involved in either stimulating or preventing 25 apoptosis, also supports a possible application for bromelain to prevent apoptosis.

30 Prevention of parasite/pathogen invasion and survival in cells.

The invasion of parasites and pathogens, and subsequent survival in cells, is dependent on these organisms utilising host cell signalling pathways (Bliska et al,

Cell. 73: 903-920 (1993)). In particular, *Salmonella* has been demonstrated to phosphorylate MAP kinase which allows for the bacteria to become endocytosed by macrophages (Galan et al, Nature 357: 588-589 (1992)).
5 The bacteria then proliferate and destroy the cell. Because bromelain has been shown to modify host signalling pathways (Figure 13a), and in particular inhibit tyrosine phosphorylation of MAPkinase (Figure 2), we believe that another potential application for
10 bromelain could be to inhibit either parasite/pathogen invasion or their survival in cells.

CLAIMS

1. The use of bromelain in the preparation of an agent for modulating intracellular signal pathways which depend upon inositol phosphates, protein kinases and/or protein phosphatases.
2. The use as claimed in claim 1 wherein the signal pathway depends upon inositol phosphates.
- 10 3. The use as claimed in claim 1 or claim 2 wherein the agent modulates pathways controlling cell growth and proliferation.
- 15 4. The use as claimed in claim 3 in the preparation of an agent for reducing or preventing the production of growth factors by cells.
5. The use as claimed in claim 4 in the preparation of an agent for preventing the formation of MAP kinase.
- 20 6. The use of bromelain in the preparation of an agent for the treatment or control of a disease mediated by inositol phosphate, protein kinase and/or protein phosphatase -mediated intracellular signal transduction.
- 25 7. The use of bromelain in the preparation of an agent for modulating cytokine production.
- 30 8. The use as claimed in claim 7 wherein the agent stimulates cytokine production.
9. The use as claimed in claim 8 wherein the agent is

used as an immunoenhancer and/or as an adjuvant for a vaccine.

10. The use as claimed in claim 7 wherein the agent
5 inhibits cytokine production.

11. The use as claimed in claim 10 wherein the agent is
an immunosuppressor.

10 12. The use as claimed in claim 11 wherein the agent is
for use in preventing or treating tissue rejection, or to
prevent autoimmune responses.

13. The use as claimed in claim 10 wherein the agent is
15 for use in preventing or treating toxic shock.

14. The use of bromelain in the preparation of an agent
for the treatment or prevention of an autoimmune disease
or transplant rejection by a host.

20 15. The use as claimed in claim 14, wherein the
autoimmune disease is multiple sclerosis or rheumatoid
arthritis.

25 16. The use of bromelain in the preparation of an agent
for use in preventing or treating toxic shock.

17. The use of bromelain in the preparation of an agent
for use as an adjuvant for a vaccine.

30 18. The use of bromelain in the preparation of an agent
for the treatment of cancer.

19. The use of bromelain in the preparation of an agent for use in the prevention or treatment of allergies.
- 5 20. A method for the treatment or prevention of an autoimmune disease, the method comprising administering to a patient an effective amount of bromelain.
- 10 21. A method for the treatment or prevention of transplant rejection, the method comprising administering to a patient an effective amount of bromelain.
- 15 22. A method for the treatment or prevention of toxic shock, the method comprising administering to a patient an effective amount of bromelain.
- 20 23. A method for inducing immunostimulation, the method comprising administering to patient an effective amount of bromelain together with a vaccine.
- 25 24. The use of bromelain in the preparation of an agent to prevent apoptosis.
- 25 25. A method of preventing apoptosis, the method comprising administering to a patient an effective amount of bromelain.
- 30 26. The use of bromelain in the preparation of an agent for use in inhibiting, preventing or treating parasite and/or pathogen infection.
- 30 27. A method of inhibiting, preventing or treating parasite and/or pathogen infection, the method comprising administering to a patient an effective amount of

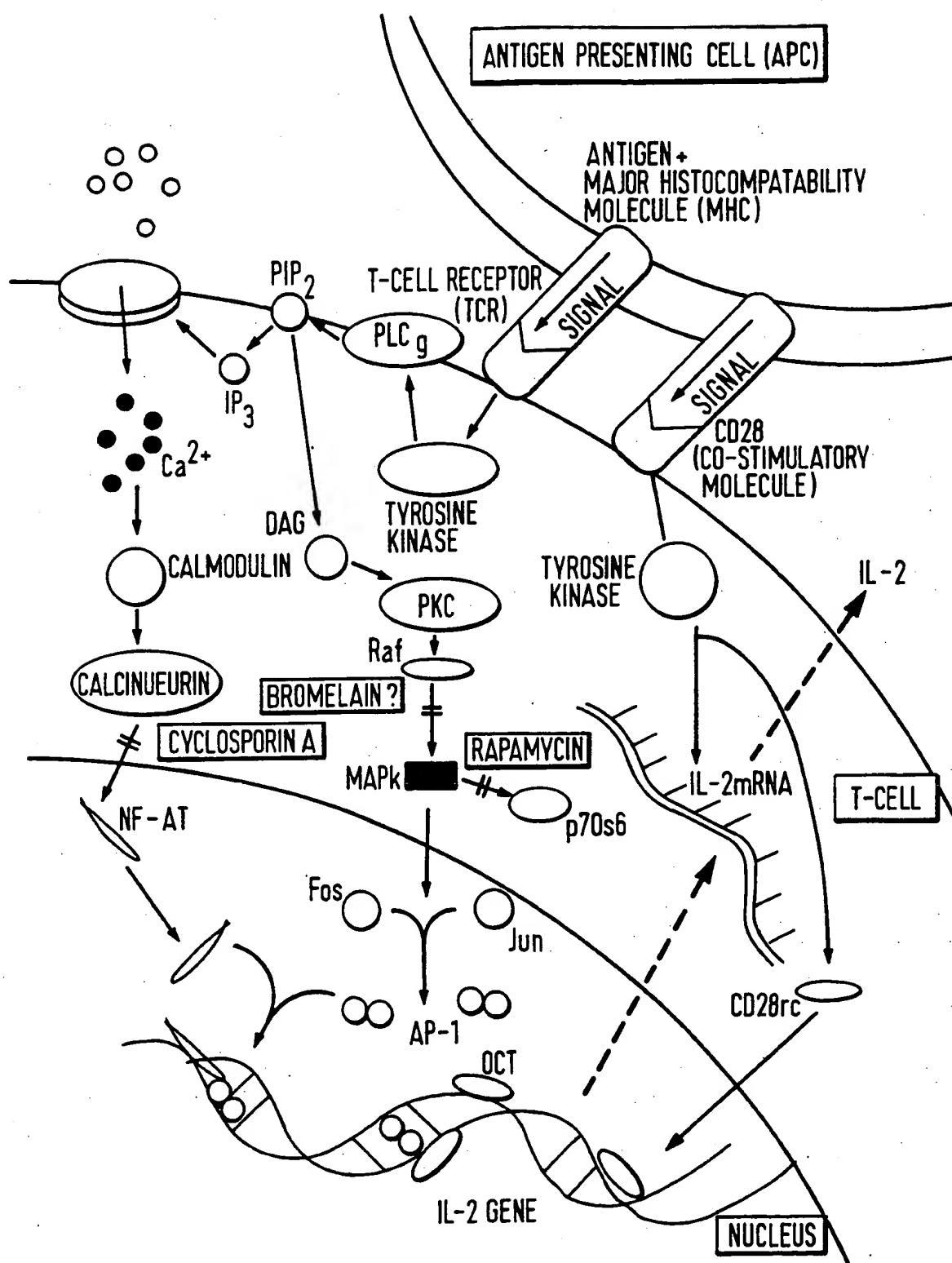
bromelain.

28. A method for the treatment of cancer, the method comprising administering to a patient an effective amount
5 of bromelain.

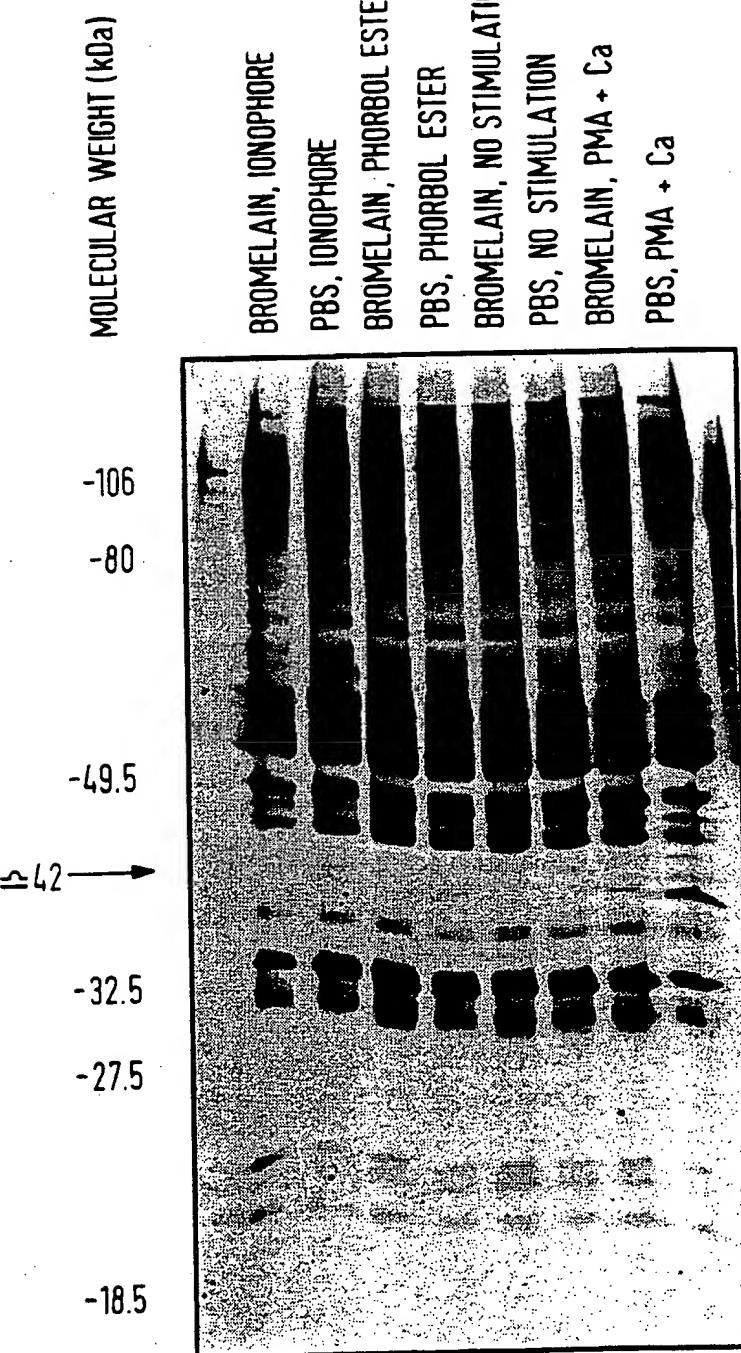
29. A method for the prevention or treatment of allergies, the method comprising administering to a patient an effective amount of bromelain.

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FIG. 1

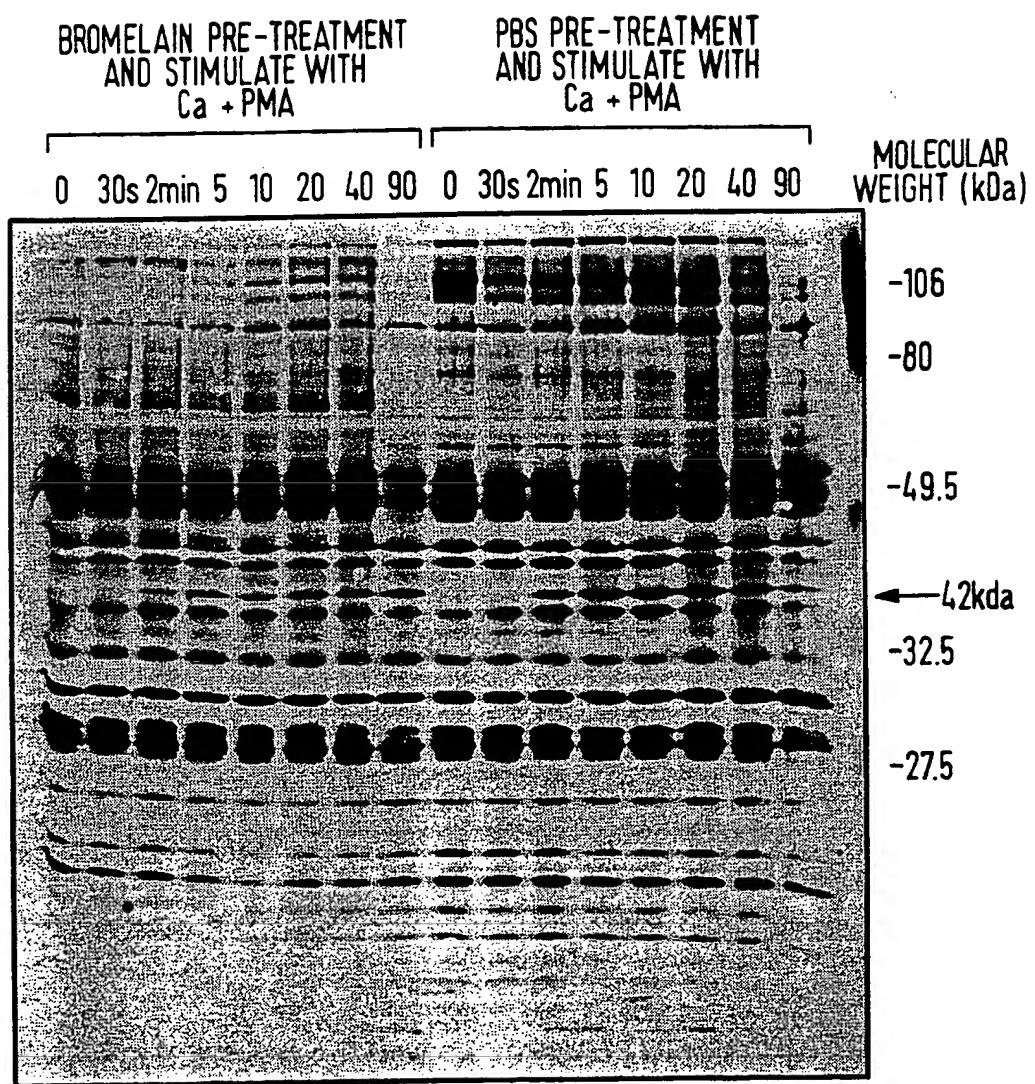


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FIG. 2

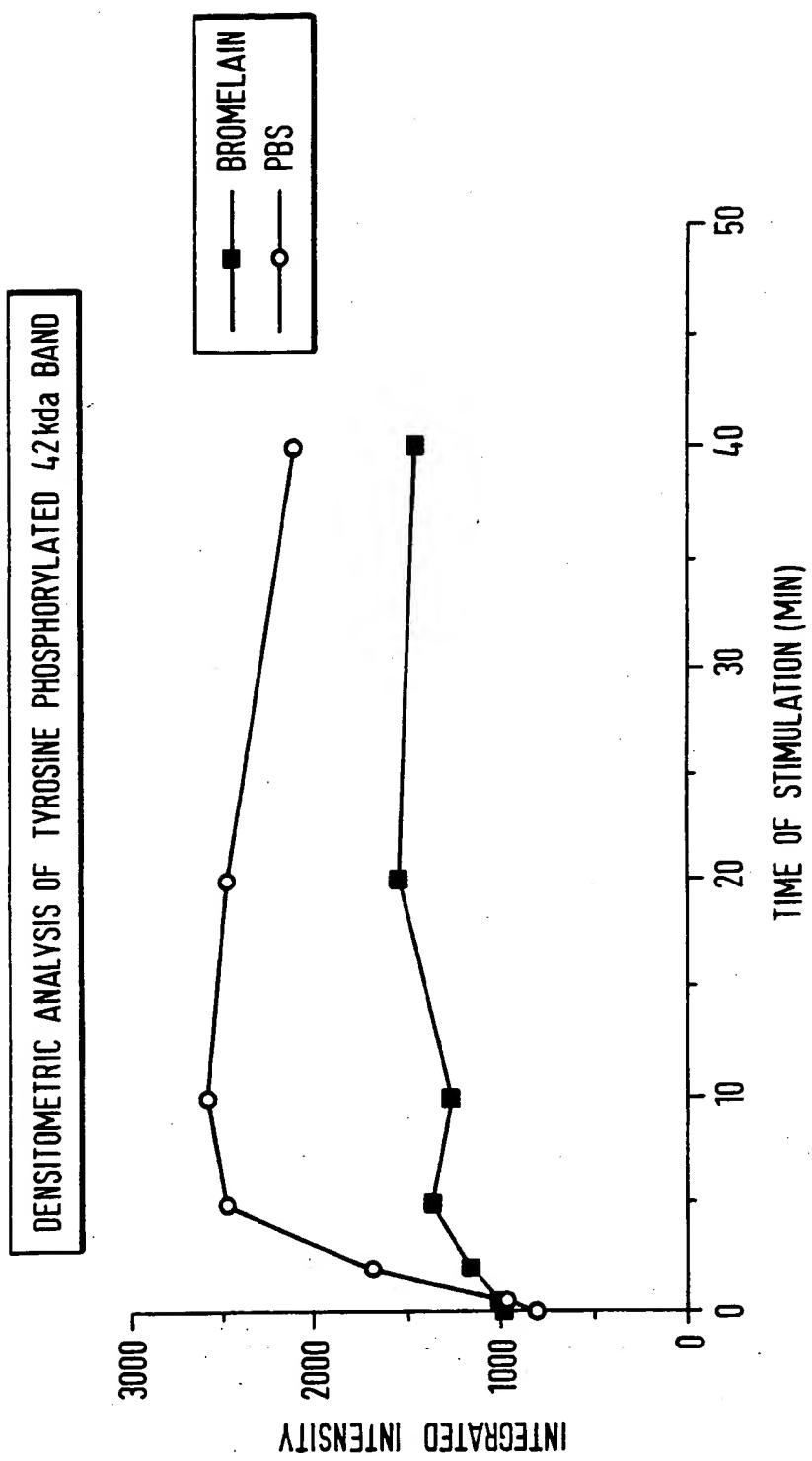
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FIG. 3



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FIG. 4



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FIG. 5

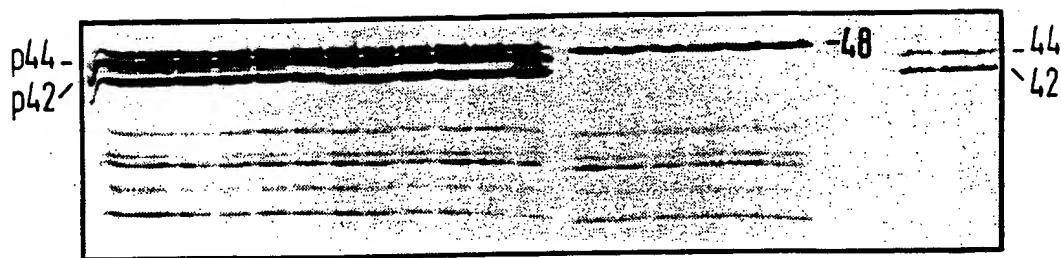


FIG. 6

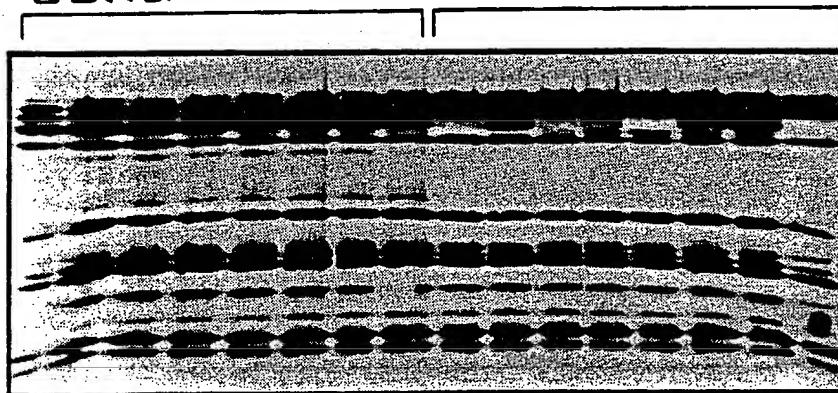
1 MIN
#7b

BROMELAIN, PMA + Ca

PBS, PMA + Ca

TIME (MIN)

0.5 2 5 10 20 40 90 180



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FIG. 7

PMA + CALCIUM IONOPHORE A23187

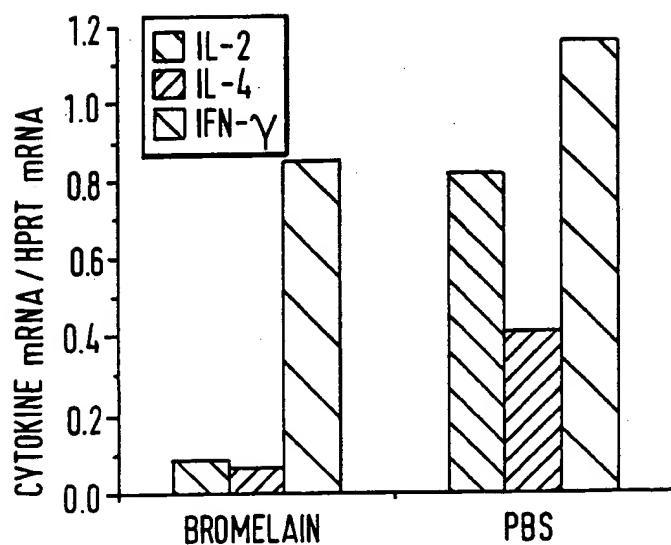
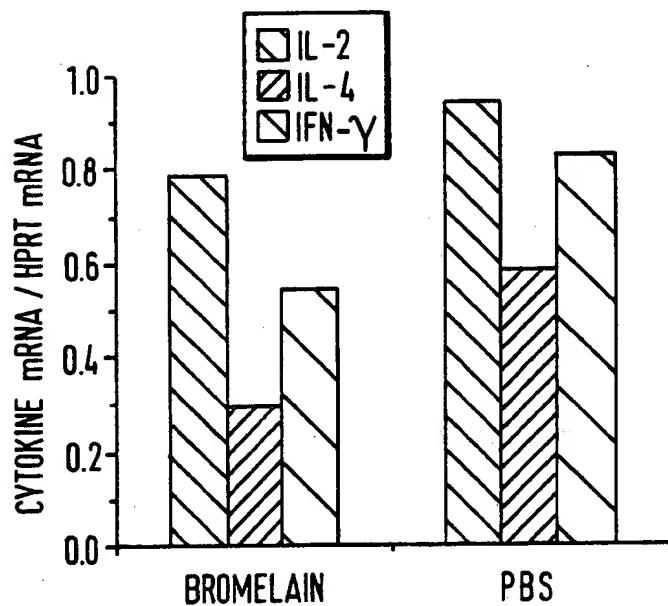
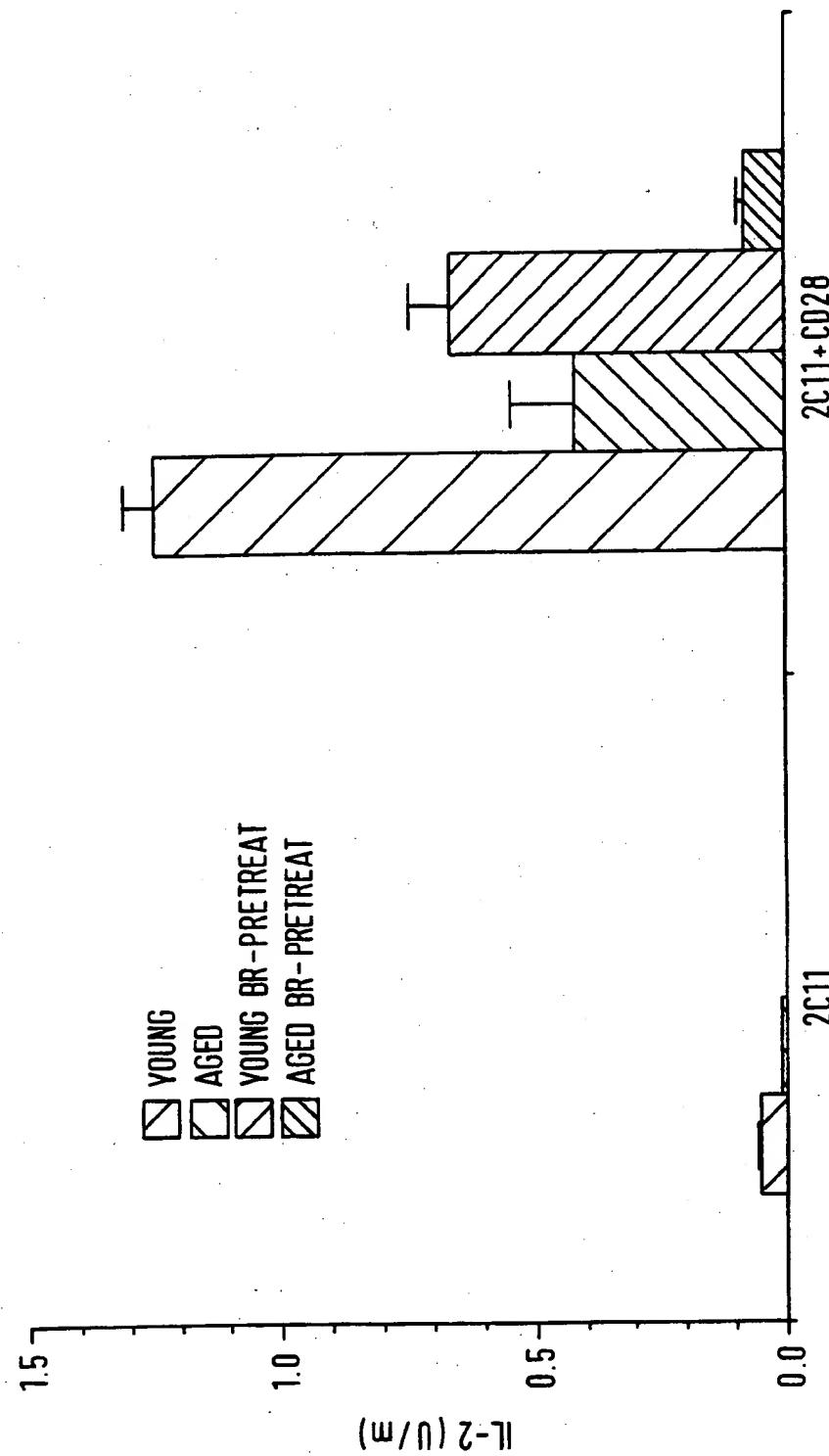


FIG. 9



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FIG. 8
IL-2 PRODUCTION BY T CELLS FOLLOWING 24 HOURS OF STIMULATION



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FIG. 10

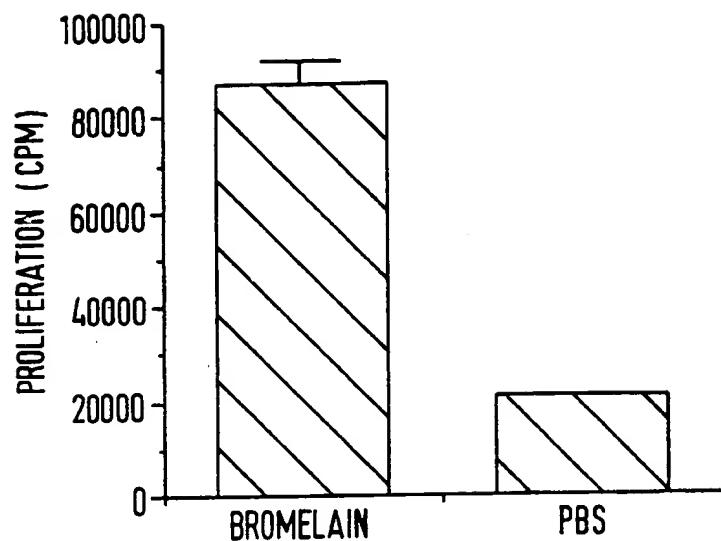
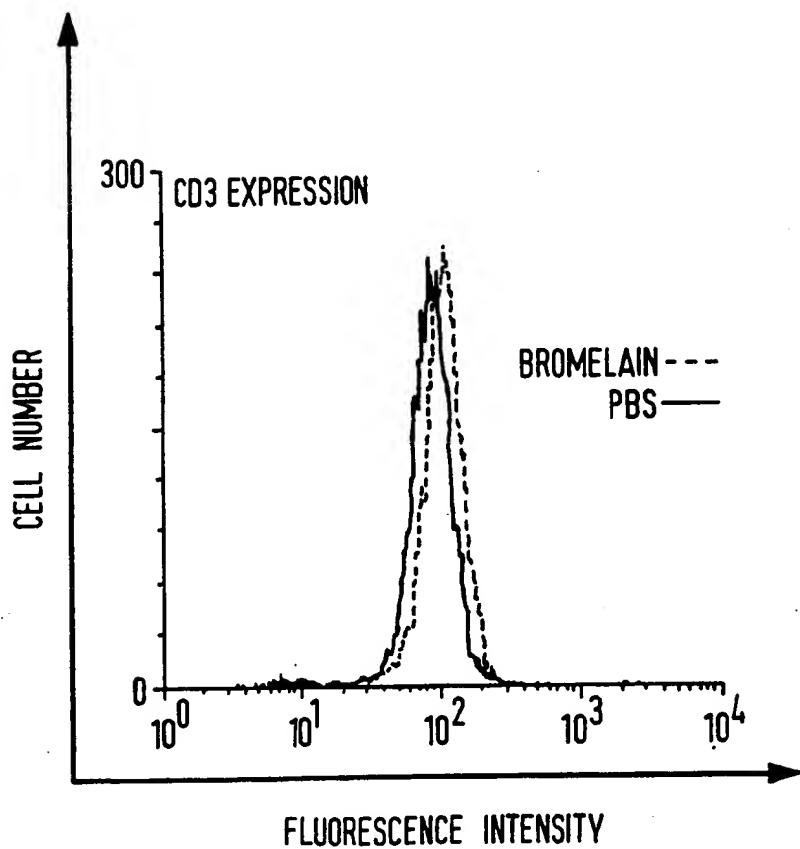
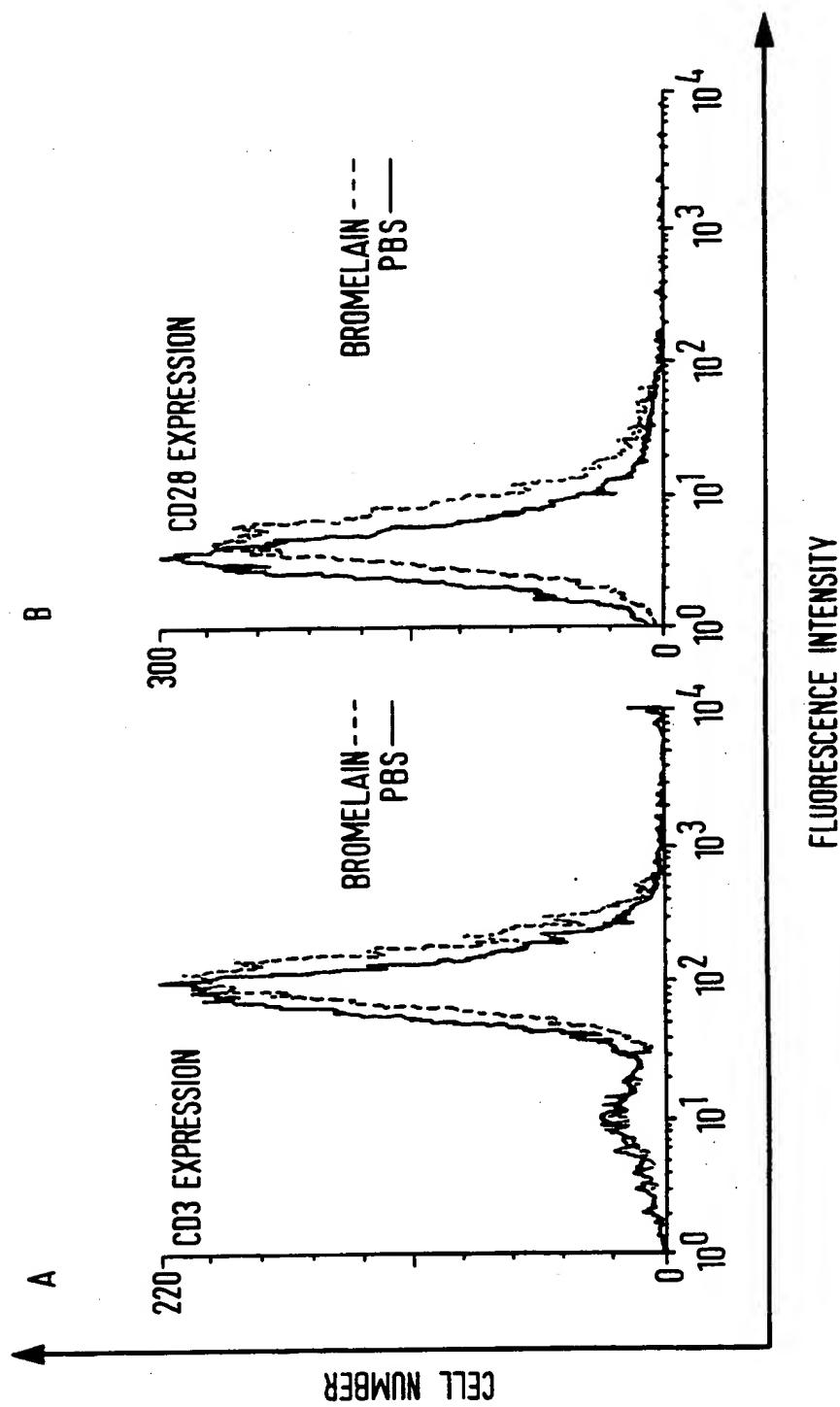


FIG. 12



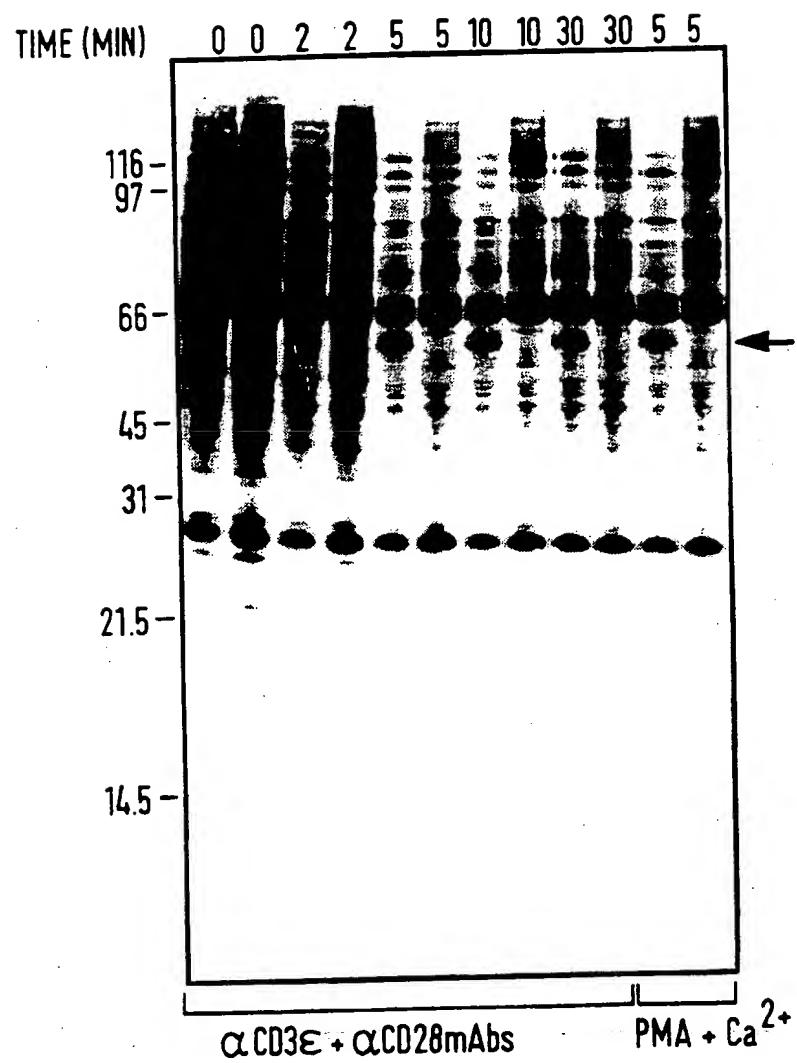
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FIG. 11



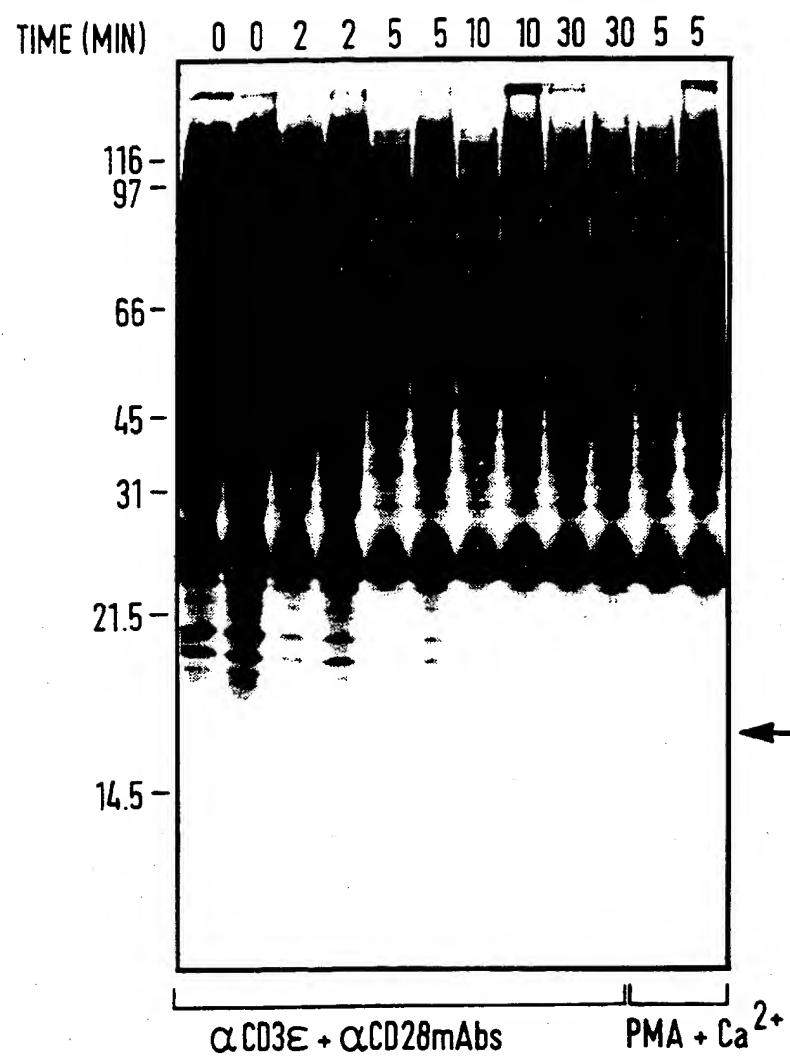
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FIG. 13a



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FIG. 13b



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FIG. 14

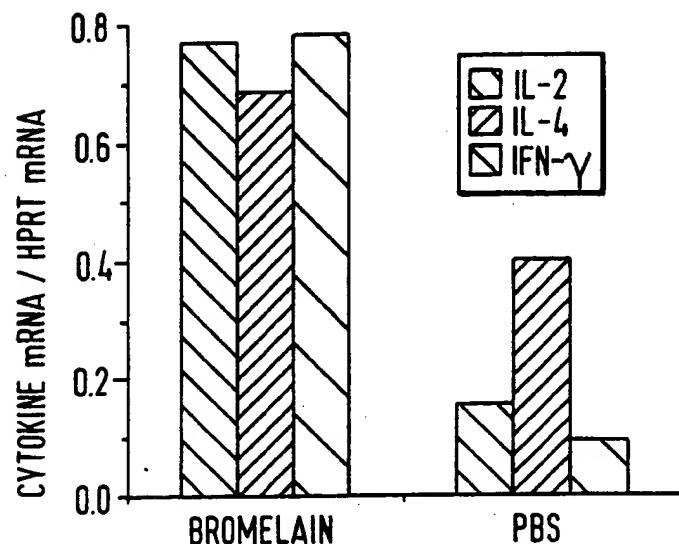
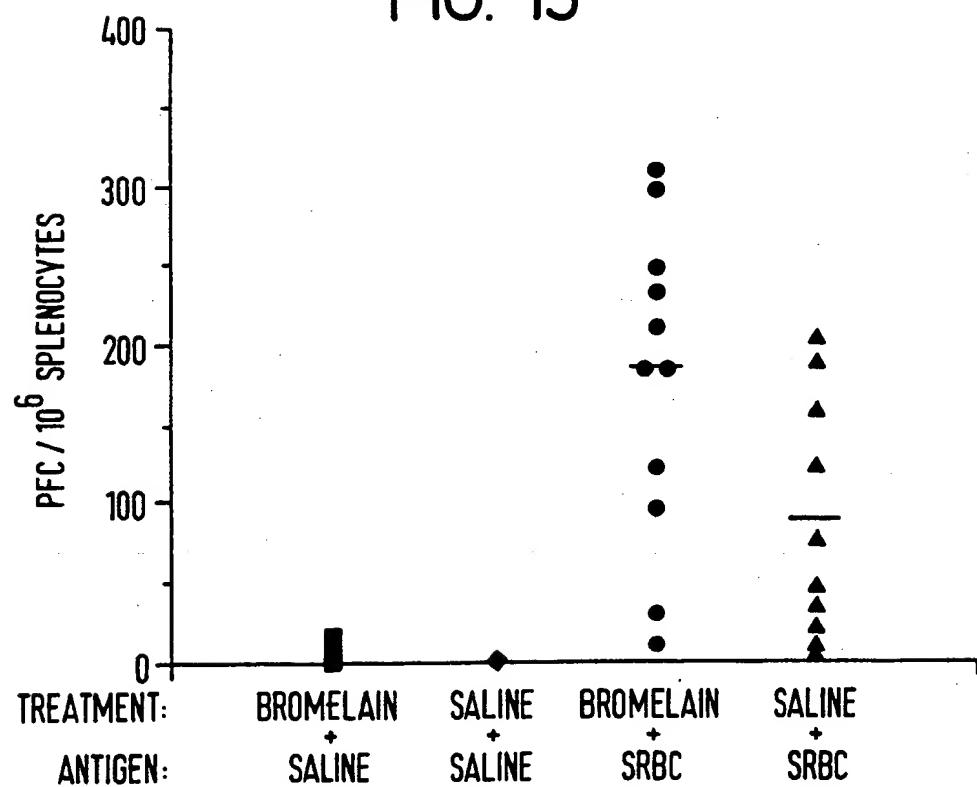
ANTI-CD3 ε + ANTI-CD28 mAbs

FIG. 15



INTERNATIONAL SEARCH REPORT

Inte: mal Application No
PCT/GB 95/01501A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANTA MEDICA, 1988 pages 377 - 381 MAURER H. R. ET AL. 'BROMELAIN INDUCES THE DIFFERENTIATION OF LEUKEMIC CELLS IN VITRO.' cited in the application see the whole document ---	1-29
X	US,A,5 223 406 (KARL RANSBERGER ET AL.) 29 June 1993 see column 1, line 1 - column 1, line 52 ---	1-29 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'B' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

1

Date of the actual completion of the international search

3 October 1995

Date of mailing of the international search report

16.11.95

Name and mailing address of the ISA

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 Fax: (+ 31-70) 340-3016

Authorized officer

Rempp, G

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 8505, Derwent Publications Ltd., London, GB; Class B04, AN 85-028979 & JP,A,59 225 122 (KAKEN PHARM CO LTD) 18 December 1984 see abstract</p> <p>---</p>	1,3,18, 28
X,P	<p>DE,A,43 02 060 (MUCOS PHARMA GMBH) 28 July 1994 see column 1, line 1 - column 3, line 20</p> <p>---</p>	1-29
X,P	<p>WO,A,95 00169 (CORTECS LIMITED) 5 January 1995 see page 5, line 16 - page 13, line 29</p> <p>-----</p>	1-29

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/GB95/01501**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: although claims 20-23, 25, 27-29 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 95/01501

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5223406	29-06-93	EP-A-	0421023	10-04-91
		AT-T-	119780	15-04-95
		DE-D-	58909121	20-04-95
DE-A-4302060	28-07-94	NONE		
WO-A-9500169	05-01-95	AU-B-	7002794	17-01-95

